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(57) Abstract

This invention provides a method for detecting in a sample an enzyme which catalyzes the synthesis of a double-stranded nucleic acid molecule from a nucleic acid template which comprises contacting the sample with a suitable fluorophore which selectively binds to double-stranded DNA. Also provided is a method for determining in a sample the activity of such an enzyme. This invention also provides a method for detecting an RNA-DNA heteroduplex in a sample which comprises contacting the sample with a suitable fluorophore which selectively binds to double-stranded DNA. This method for detecting an RNA-DNA heteroduplex may further comprise quantitatively determining detected RNA-DNA heteroduplex. This invention also provides a method for detecting in a sample en enzyme which catalyzes the synthesis of and RNA-DNA heteroduplex from a nucleic acid template, as well as a method of detecting the activity of such an enzyme in a sample. This invention further provides a method for detecting the viral load of HIV in a sample. Also provided is a method for diagnosing an HIV infection in a subject, and for monitoring the progression of an HIV infection in a subject. Also provided is a method for determining the viral load of HIV in a subject infected with HIV. This invention further provides a method for identifying whether a substance inhibits reverse transcriptase. Finally, this invention provides a kit for assaying an enzyme which catalyzes the synthesis of a double-stranded nucleic acid molecule from a nucleic acid template.

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METHOD AND KIT FOR FLUOROMETRIC ANALYSIS OF ENZYMES CATALYZING SYNTHESIS OF NUCLEIC ACIDS

This application is a continuation of U.S. Serial No. 08/386,469, filed February 10, 1995, the contents of which are hereby incorporated by reference into the present application.

Throughout this application various publications are referenced by arabic numerals within parentheses. Full citations for these publications may be found at the end of this application, preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

Background of the Invention

The epidemic of human immunodeficiency virus (HIV) infection and AIDS continues to grow in the United States and world-wide (25). Although an enormous body of basic information concerning viral pathogenesis and life cycle has been described (26,27), mortality due to AIDS continues to rise.

With better understanding of the HIV life cycle and HIV-host cell interactions have come strategies for interrupting viral replication (28). Theoretically, any step in the HIV life cycle is susceptible to pharmacologic attack and many targets for antiviral intervention have been investigated (29). The virus-encoded reverse transcriptase (RT) remains the most accessible of the targets under investigation (23). To date, four nucleoside analog inhibitors of reverse transcription have received approval for marketing in the United States, with other nucleoside and non-nucleoside compounds in advanced clinical trials. The search for potent inhibitors of RT continues to be a major focus of research. In addition, although p24 antigen levels in culture supernatants is routinely used as a surrogate marker for viral replication in in vitro culture systems for screening inhibitors of viral replication, RT activity in culture supernatants has and continues to be used as a surrogate marker of viral load (30,7).

We have developed a rapid, efficient, and inexpensive assay for measuring RT activity using a novel fluorometric approach. All antiretrovirals currently approved for use in the United States for the treatment of human immunodeficiency virus type 1 (HIV-1) infection are inhibitors of RT (1), and the search for potent inhibitors of this enzyme continues to be a major focus of research. Although conventional isotopic methods for RT activity are highly sensitive, they all suffer from the following significant disadvantages which limit their utility: (a) the performance of these assays is limited to laboratories that hold appropriate licenses for radioactivity; (b) they are highly labor-intensive and time-consuming procedures; (c) the disposal of large quantities of scintillation fluid and radioactive isotopes required for these assays is a growing environmental concern; and (d) there are many sources of potential errors since the assays are complex (these procedures involve steps wherein the RNA-DNA heteroduplex produced by RT catalysis is precipitated or bound to filters and washed free of the radiolabeled substrate). Nonradiometric assays that measure incorporation of nucleotides by RT into DNA by immunological methods (2-5) do not suffer these disadvantages. Unfortunately, commercially available kits based on these immunological detection methods are very costly (~$400 per 96-well plate).

Summary of the Invention

This invention provides a method for detecting in a sample an enzyme which catalyzes the synthesis of a double-stranded nucleic acid molecule from a nucleic acid template which comprises: (a) treating the sample under conditions sufficient to initiate catalysis by the enzyme; (b) contacting the sample with a suitable fluorophore which selectively binds to double-stranded DNA under conditions permitting said fluorophore to bind to double-stranded nucleic acid molecules in the sample synthesized by the catalysis; and (c) detecting the presence of fluorescence in the sample resulting from step (b), thereby detecting in the sample the enzyme.

This invention also provides a method for determining in a sample the activity of an enzyme which catalyzes the synthesis of a double-stranded nucleic acid molecule from a nucleic acid template which comprises: (a) treating the sample under conditions sufficient to initiate catalysis by the enzyme; (b) contacting the sample with a suitable fluorophore which selectively binds to double-stranded DNA under conditions permitting said fluorophore to bind to double-stranded nucleic acid molecules in the sample synthesized by the catalysis; (c) quantitatively measuring fluorescence in the sample resulting from step (b); and (d) calculating the activity of any enzyme in the sample as a predetermined function of the quantity of fluorescence measured in step (c).

The subject invention also provides a method for detecting an RNA-DNA heteroduplex in a sample which comprises: (a) contacting the sample with a suitable fluorophore which selectively binds to double-stranded DNA under conditions permitting said fluorophore to bind to the RNA-DNA heteroduplex in the sample; and (b) detecting the presence of fluorescence in the sample resulting from step (a).

The subject invention also provides the aforementioned method of detecting an RNA-DNA heteroduplex in a sample, which method further comprises quantitatively measuring fluorescence detected in step (b) so as to quantitatively determine the RNA-DNA

heteroduplex in the sample.

The subject invention further provides a method for detecting in a sample an enzyme which catalyzes the synthesis of an RNA-DNA heteroduplex from a nucleic acid template which comprises: (a) treating the sample under conditions sufficient to initiate catalyses of RNA-DNA heteroduplexes by the enzyme; and (b) detecting the presence of RNA-DNA heteroduplexes in the sample resulting from step (a) according to the aforementioned method of detecting an RNA-DNA heteroduplex in a sample, thereby detecting in the sample the enzyme.

The subject invention further provides a method for determining in a sample the activity of an enzyme which catalyzes the synthesis of an RNA-DNA heteroduplex from a nucleic acid template which comprises: (a) treating the sample under conditions sufficient to initiate catalyses of RNA-DNA heteroduplexes by the enzyme; (b) quantitatively determining RNA-DNA heteroduplexes in the sample resulting from step (a) according to the aforementioned method of quantitatively determining an RNA-DNA heteroduplex in a sample; and (c) calculating the activity of any enzyme in the sample as a predetermined function of the quantity of RNA-DNA heteroduplex determined in step (b).

This invention also provides a method for determining in a sample viral load of HIV which comprises: (a) determining the activity of reverse transcriptase in the sample according to the aforementioned method of determining in a sample the activity of an enzyme which catalyzes the synthesis of an RNA-DNA heteroduplex from a nucleic acid template; and (b) calculating the viral load of HIV in the sample as a predetermined function of the activity of reverse transcriptase determined in step (a).

The subject invention also provides a method for diagnosing an HIV infection in a subject which comprises: (a) obtaining a suitable sample from the subject; and (b) detecting the presence of reverse transcriptase in the sample according to the aforementioned method for detecting in a sample an enzyme which catalyzes the synthesis of an RNA-DNA heteroduplex from a nucleic

acid template, the presence of reverse transcriptase indicating an HIV infection.

The subject invention further provides a method for determining the viral load of HIV in a subject infected with HIV which comprises: (a) obtaining a suitable sample from the subject; and (b) determining the viral load of HIV in the sample according to the aforementioned method for determining in a sample viral load of HIV, thereby determining the viral load in the subject.

The subject invention further provides a method for monitoring over a period of time the progression of an HIV infection in a subject infected with HIV which comprises: (a) determining the viral load of HIV in the subject according to the aforementioned method for determining the viral load of HIV in a subject at a plurality of points suitably spaced over the period of time, thereby determining a plurality of viral loads; and (b) comparing the viral loads determined in step (a), thereby monitoring the progression of the HIV infection in the subject over the period of time.

The subject invention also provides a method for identifying whether a substance inhibits reverse transcriptase which comprises: (a) obtaining a sample comprising reverse transcriptase, the activity of the reverse transcriptase in the sample being predetermined; (b) contacting the sample with the substance; (c) determining the activity of reverse transcriptase in the sample resulting from step (b) according to the aforementioned method for determining in a sample the activity of an enzyme which catalyzes the synthesis of an RNA-DNA heteroduplex from a nucleic acid template; and (d) ascertaining whether the activity determined in step (c) is less than the predetermined activity of reverse transcriptase in the sample obtained in step (a), a lower activity in step (c) indicating inhibition of reverse transcriptase by the substance.

Finally, this invention provides a kit for assaying an enzyme which catalyzes the synthesis of a double-stranded nucleic acid molecule from a nucleic acid template which comprises: (a)

substrates for initiating catalysis by the enzyme; and (b) a suitable fluorophore capable of selectively binding to double-stranded DNA.

Brief Description of the Drawings

Figure 1. Dependence of DAPI fluorescence on the amount of HIV-1 RT. Purified recombinant HIV-1 RT was added in the indicated quantities to the RT assay described in the "Experimental Details" Section and incubated from 0 to 60 minutes at 37°C before stopping the reaction with EDTA. Aliquots were dispensed into cuvettes containing DAPI, and the resulting fluorescence was measured after 30 minutes incubation. The rate of fluorescence increase was linearly dependent on the amount of HIV-1 RT added to the RT assay up to 80 ng (r^2 = 0.9997; inset).

Figure 2. Comparison of the fluorometric RT assay with an isotopic assay for RT activity. The fluorometric and isotopic assays were performed as described in the "Experimental Details" Section at the same time with the same preparation of purified recombinant HIV-1 RT. The concentration of the stock RT from which the dilutions was prepared was 0.5 mg/ml. The specific activity of RT from the experiment shown is calculated to be 630 ± 56 nmol dTTP incorporated/min/mg protein, and is consistent with the specific activity of our purified RT preparations. Both assays are highly correlated to each other over the same range of dilutions (1:250-1:5000) of RT (r^2 = 0.986).

Figure 3. Stability of the fluorometric RT assay as a function of time. Pooled active fractions from the purification of RT (Table 1) were diluted 1:1000-1:5000 and were assayed in duplicate for RT activity by the fluorometric RT assay. In addition to assessing the fluorescence at 30 minutes incubation with DAPI, the cuvettes were kept in the dark and the fluorescence was reassessed at the time points indicated. The fluorescence at each time point was normalized to

the fluorescence determined at 30 minutes, and the mean ± SE percentage fluorescence, represented by the symbols, was determined.

Figure 4. Effect of AZT 5'-triphosphate, nevirapine, and oltipraz on HIV-1 RT activity as measured by DAPI fluorescence. Assays were carried out as described in the "Experimental Details" Section. Top, plot of 1/velocity as a function of AZT 5'-triphosphate appears to indicate competitive inhibition of HIV-1 RT. This is confirmed by the negligible y-intercept of the corresponding Segel plot (inset; $r^2 = 0.999$). The K_i was determined to be 13.8 nM for the experiment shown. Middle, plot of 1/velocity as a function of nevirapine concentration appears to show noncompetitive inhibition with respect to dTTP. However, the Segel transformation (inset; $r^2 = 0.997$) of these data indicates that the inhibition is mixed (α=0.76), and the K_i was determined to be 25.8 μM. The two other experiments with nevirapine also showed mixed inhibition. Bottom, time- and concentration-dependent inhibition of HIV-1 RT by oltipraz. The Kitz-Wilson transformation is shown in the inset ($r^2 = 0.874$). The K_i and k_3 for this experiment was determined to be 28.5 μM and 0.074 h^{-1}, respectively.

Detailed Description of the Invention

This invention provides a method for detecting in a sample an enzyme which catalyzes the synthesis of a double-stranded nucleic acid molecule from a nucleic acid template which comprises: (a) treating the sample under conditions sufficient to initiate catalysis by the enzyme; (b) contacting the sample with a suitable fluorophore which selectively binds to double-stranded DNA under conditions permitting said fluorophore to bind to double-stranded nucleic acid molecules in the sample synthesized by the catalysis; and (c) detecting the presence of fluorescence in the sample resulting from step (b), thereby detecting in the sample the enzyme.

In the subject method, the fluorophore detects an enzyme which catalyzes the synthesis of a double-stranded nucleic acid molecule from a nucleic acid template by binding to the product of the catalysis, i.e. by binding to the double-stranded nucleic acid molecule synthesized by the addition of nucleotides or nucloesides to the nucleic acid template. The nucleic acid template may be any single-stranded nucleic acid molecule, such as single-stranded RNA or DNA. The nucleic acid template may be a naturally-occurring nucleic acid, for example DNA found in a cell, or it may be a synthetic nucleic acid, such as synthetic poly-dT or poly-A. The double-stranded nucleic acid may thus be a double-stranded DNA molecule, a double-stranded RNA molecule, or an RNA-DNA heteroduplex.

Enzymes which catalyze the synthesis of a double-stranded nucleic acid molecule from a nucleic acid template are well-known to those of ordinary skill in the art, and any such enzyme may be detected by the subject method. Examples of enzymes which catalyze the synthesis of a double-stranded nucleic acid molecule from a nucleic acid template include, but are not limited to, DNA polymerases, such as reverse transcriptase (RT) of DNA Polymerase I, which catalyze the synthesis of a DNA molecule from a DNA template; and RNA polymerase, which catalyzes the synthesis of an RNA molecule from a DNA template.

Any fluorophore which is capable of binding to double-stranded

DNA may be used in the subject invention. In a double-stranded DNA molecule, such fluorophores bind to the minor groove of the molecule. Positively charged regions on the fluorophore are thought to bind to the negatively charged phosphate backbone in the minor groove. Binding to the minor groove prevents the transfer of a proton to the fluorophore which occurs in solution and quenches fluorescence. Prevention of this proton transfer causes these fluorophores to fluoresce. Without limiting the subject invention, it is believed that such fluorophores also bind to a minor groove in RNA-DNA heteroduplexes, resulting in fluorescence. Fluorophores which bind to double-stranded DNA molecules are well known in the art and include, but are not limited to, 4',6-diamidino-2-phenylindole; acridine orange; acridine homodimer; acridine-ethidium heterodimer; 9-amino-6-chloro-2-methoxyacridine; aminoactinomycin-D; benzothiazolium-4-quinolinium dimer dyes; bisbenzamide dyes; and ethidium homodimer.

The fluorophore used in the subject method must be suitable for binding to the particular double-stranded nucleic acid molecule synthesized by the catalysis. Determination of suitable fluorophores for binding to a particular nucleic acid molecule is well known to those of ordinary skill in the art. The suitability of a fluorophore for binding to a particular double-stranded nucleic acid molecule depends on such known factors as the base composition of the double-stranded nucleic acid molecule. The composition of the double-stranded nucleic acid molecule in turn depends of the composition of the nucleic acid template used as a substrate for initiation of catalysis. For example, 4',6-diamidino-2-phenylindole (DAPI) preferentially binds to contiguous dA-dT base pairs. Accordingly, DAPI is particularly suitable for use in a method as described above in which a nucleic acid template comprised of contiguous A-T base pairs is used as a substrate for initiation of catalysis of double-stranded nucleic acid. Certain fluorophores are, however, known to be less preferential in their binding and are known to bind a double-stranded DNA molecule regardless of the molecule's composition.

In the method of the subject invention, fluorescence may be detected using any known method. For example, fluorescence may be detected by visually assessing the sample for the presence of fluorescent light. Preferably, however, fluorescence is detected by means of a fluorometer.

For purposes of the subject invention, a sample is any sample in which the detection of an enzyme which catalyzes the synthesis of a double-stranded nucleic acid molecule from a nucleic acid template is desired. In one embodiment, the sample is a biological fluid. Biological fluids are well-known to those of ordinary skill in the art and include, but are not limited to, urine, semen, saliva, and fluid blood fractions such as serum or plasma. Biological fluids also include tissue culture supernatants, such as the supernatant from cultured peripheral blood mononuclear cells or from cell lines. In another embodiment, the sample is a tissue. Examples of tissue samples which may be used in the method of the subject invention include samples lymphoid organs, such as lymph nodes or spleen; and tissue fractions of blood, such as lymphocytes. For purposes of the subject invention, the sample may already be predetermined to comprise the enzyme, such as a sample of the purified enzyme. Conversely, it may be unknown whether or not the sample contains the enzyme.

In one embodiment of the method of the subject invention, the sample is obtained from a mammal. When the sample is obtained from a mammal, in a further embodiment the sample is obtained from a human.

Any conditions sufficient to initiate catalysis by the enzyme may be used to treat the sample in step (a) of the subject method. Such conditions are well-known to those of ordinary skill in the art and will depend on the particular enzyme whose detection is desired. For example, treating the sample in step (a) may comprise contacting the sample with substances such as substrates and cofactors required by the enzyme to catalyze synthesis of the product double-strand nucleic acid molecule. In the case of DNA polymerases, such as RT, such substances include a nucleic acid

template, a primer molecule, deoxynucleoside triphosphates, magnesium, and manganese salts. In the case of RNA polymerase, such substances include, a nucleic acid template, ribonucleotide triphosphates, and magnesium.

In one embodiment of the subject method, the method further comprises treating the sample resulting from step (a) under conditions sufficient to terminate catalysis by the enzyme prior to contacting the sample with the fluorophore. Treatments useful for termination of catalysis are well-known to those of ordinary skill and may depend on the particular enzyme whose detection is desired. Such treatments include, but are not limited to, contacting the sample with EDTA, contacting the sample with an inhibitor of the enzyme, or diluting the sample to a concentration at which reaction between the substrates and the nucleic acid template cannot occur.

This invention also provides a method for determining in a sample the activity of an enzyme which catalyzes the synthesis of a double-stranded nucleic acid molecule from a nucleic acid template which comprises: (a) treating the sample under conditions sufficient to initiate catalysis by the enzyme; (b) contacting the sample with a suitable fluorophore which selectively binds to double-stranded DNA under conditions permitting said fluorophore to bind to double-stranded nucleic acid molecules in the sample synthesized by the catalysis; (c) quantitatively measuring fluorescence in the sample resulting from step (b); and (d) calculating the activity of any enzyme in the sample as a predetermined function of the quantity of fluorescence measured in step (c).

As used herein, the activity of an enzyme in a sample is the number of reactions catalyzed in the sample per unit time. Enzymatic activity in a sample depends on factors including, but not limited to, the number of enzyme molecules present in the sample. As is known in the art, the number of reactions catalyzed per unit time may be evaluated by analyzing a variety of parameters. In the method of the subject invention, the number of reactions catalyzed per unit time is evaluated by

analyzing the quantity of product, i.e. double-stranded nucleic acid, produced per unit time.

Accordingly, in the subject method, the activity of the enzyme is determined as a function of the quantity of fluorescence measured in step (c), since the quantity of fluorescence indicates the quantity of fluorescing fluorophore molecules bound to double-stranded nucleic acid molecules in the sample. The functional relationship between the activity of the enzyme and the quantity of fluorescence may be arbitrary. For example, the enzymatic activity may be arbitrarily deemed to be equivalent to the quantity of the fluorescence. Alternatively, a quantity of fluorescence may be converted to enzymatic activity in terms of, for example, moles substrate consumed by generating a calibration curve of fluorescence with an enzyme preparation of known activity. As is known in the art, the fluorometer can be calibrated thereafter with the use of a stable fluorescent solution such as quinine sulfate.

As used herein, the quantity of fluorescence in a sample means the intensity of fluorescent light emitted by the sample. In the subject invention, the quantity of fluorescence may be quantitatively measured using any known method. For example, the fluorescence may be quantitatively measured visually by assessing the intensity of any fluorescence in the sample, the more intense the fluorescence, the greater the quantity of fluorophore bound to double-stranded nucleic acid molecules. Preferably, however, the fluorescence is quantitatively measured using a fluorometer.

In one embodiment of the subject method, the method further comprises treating the sample resulting from step (a) under conditions sufficient to terminate catalysis by the enzyme prior to contacting the sample with the fluorophore. Treatments sufficient to terminate catalysis of an enzyme in a sample are well-known to those of ordinary skill in the art and are described above.

The subject invention also provides a method for detecting an RNA-DNA heteroduplex in a sample which comprises: (a) contacting

the sample with a suitable fluorophore which selectively binds to double-stranded DNA under conditions permitting said fluorophore to bind to any RNA-DNA heteroduplex in the sample; and (b) detecting the presence of fluorescence in the sample resulting from step (a).

Any fluorophore capable of selectively binding to double-stranded DNA may be used in the subject method for detecting RNA-DNA heteroduplexes present in a sample, and such fluorophores are well-known to those of ordinary skill in the art. In different embodiments, the fluorophore in the subject method is 4',6-diamidino-2-phenylindole; acridine orange; acridine homodimer; acridine-ethidium heterodimer; 9-amino-6-chloro-2-methoxyacridine; aminoactinomycin-D; a benzothiazolium-4-quinolinium dimer dye; a bisbenzamide dye; and ethidium homodimer.

The subject method may be used to detect RNA-DNA heteroduplexes in any sample in which detection of such heteroduplexes is desired. Samples in which RNA-DNA heteroduplexes may be detected according to the subject method include, but are not limited to, the examples of samples described above.

Detection of RNA-DNA heteroduplexes in a sample is accomplished by detecting the fluorophore bound to RNA-DNA heteroduplexes in the sample. As indicated above, binding of the fluorophore causes the fluorophore to emit fluorescent light. The fluorescence may be detected using any known method, including, but not limited to, visual detection or detection with a fluorometer.

The quantity of fluorescence depends on the quantity of fluorescing fluorophore molecules, which in turn depends on the quantity of RNA-DNA heteroduplex in the sample capable of binding the fluorophore. As used herein, the quantity of RNA-DNA heteroduplex in a sample is a function of both the number of RNA-DNA heteroduplex molecules and the length of each molecule, i.e. it corresponds to the quantity of conjugated base pairs in the sample. Accordingly, the subject invention also provides the

above-described method of detecting RNA-DNA heteroduplexes present in a sample, which method further comprises quantitatively measuring fluorescence detected in step (b) so as to quantitatively determine the RNA-DNA heteroduplex in the sample. Methods for quantitatively measuring fluorescence are well known in the art, and any such method may be used in the subject invention. Such methods are described above.

The subject invention further provides a method for detecting in a sample an enzyme which catalyzes the synthesis of an RNA-DNA heteroduplex from a nucleic acid template which comprises: (a) treating the sample under conditions sufficient to initiate catalyses of RNA-DNA heteroduplexes by the enzyme; and (b) detecting the presence of RNA-DNA heteroduplexes in the sample resulting from step (a) according to the above-described method of detecting RNA-DNA heteroduplexes present in a sample, thereby detecting in the sample the enzyme.

In one embodiment, the method further comprises treating the sample resulting from step (a) under conditions sufficient to terminate catalysis by the enzyme prior to detecting the presence of RNA-DNA heteroduplexes in the sample. Such treatments are well-known to those of ordinary skill in the art and are described above.

Any enzyme capable of catalyzing the synthesis of RNA-DNA heteroduplexes from a nucleic acid template may be detected according to the subject method. In one embodiment, the enzyme catalyzes the synthesis of an RNA-DNA heteroduplex from an RNA template, as does RT. In a different embodiment, the enzyme catalyzes the synthesis of an RNA-DNA heteroduplex from a DNA template, as in the case of RNA polymerase.

The subject invention further provides a method for determining in a sample the activity of an enzyme which catalyzes the synthesis of an RNA-DNA heteroduplex from a nucleic acid template which comprises: (a) treating the sample under conditions sufficient to initiate catalyses of the RNA-DNA heteroduplex by the enzyme; (b) quantitatively determining the RNA-DNA

heteroduplex in the sample resulting from step (a) according to the above-described method of quantitatively determining RNA-DNA heteroduplexes present in a sample; and (c) calculating the activity of any enzyme in the sample as a predetermined function of the quantity of the RNA-DNA heteroduplex determined in step (b).

As indicated above, enzymatic activity in a sample means the number of reactions catalyzed in the sample per unit time. The number of reactions catalyzed is determinable by analyzing a variety of different parameters, for example the quantity of substrates consumed per unit time or the quantity of product produced per unit time. In the subject method, the number of reactions is determined by analyzing the quantity of product, i.e. the quantity of the RNA-DNA heteroduplex, produced. Measurement is accomplished by the determination of the quantity of fluorophore bound to RNA-DNA heteroduplex in the sample via fluorescence. Any method for measuring the quantity of fluorescence may be used, and such methods are described above.

In one embodiment, the method further comprises treating the sample resulting from step (a) under conditions sufficient to terminate catalysis by the enzyme prior to quantitatively determining RNA-DNA heteroduplexes in the sample. Treatments sufficient to terminate catalysis by such enzymes are known in the art as described above, and any such treatment be used in the subject method.

In one embodiment, the enzyme whose activity is determined is reverse transcriptase. In another embodiment, the enzyme is RNA polymerase.

This invention also provides a method for determining in a sample viral load of HIV which comprises: (a) determining the activity of reverse transcriptase in the sample according to the above-described method of determining in a sample the activity of an enzyme which catalyzes the synthesis of an RNA-DNA heteroduplex from a nucleic acid template; and (b) calculating the viral load of HIV in the sample as a predetermined function of the activity

of reverse transcriptase determined in step (a).

As used herein, the term viral load means virion concentration. In the subject method for determining viral load, the viral load may be calculated as an arbitrary function of the activity of reverse transcriptase determined in step (a). For example, the viral load may arbitrarily, for purposes of comparing two or more different samples, be deemed to be equivalent to the activity of reverse transcriptase. Alternatively, the viral load may be calculated from the actual quantity of reverse transcriptase molecules calculated in the sample, since each HIV virion contains two reverse transcriptase molecules. As described above, the actual quantity of reverse transcriptase molecules in a sample may be determined by comparing the fluorescence resulting in the sample to the fluorescence produced by a standard predetermined to contain a specific quantity of reverse transcriptase molecules.

The subject invention also provides a method for diagnosing an HIV infection in a subject which comprises: (a) obtaining a suitable sample from the subject; and (b) detecting the presence of reverse transcriptase in the sample according to the above-described method for detecting in a sample an enzyme which catalyzes the synthesis of an RNA-DNA heteroduplex from a nucleic acid template, the presence of reverse transcriptase indicating an HIV infection.

For purposes of the subject invention, a suitable sample is any sample obtained from a subject in which HIV virions would be present if the subject were infected with HIV. Such samples are well known in the art and include, but are not limited to, blood fractions, such as plasma, serum, or lymphocytes.

In one embodiment, the subject is a mammal. When the subject is a mammal, the subject may be a human being.

The subject invention further provides a method for determining the viral load of HIV in a subject infected with HIV which comprises: (a) obtaining a suitable sample from the subject; and

(b) determining the viral load of HIV in the sample according to the aforementioned method for determining in a sample viral load of HIV, thereby determining the viral load in the subject.

As indicated above, a suitable sample is any sample obtained from a subject in which HIV virions would be present if the subject were infected with HIV. Suitable samples which may be used in the method of the subject invention for determining viral load of HIV in a subject are described above.

In one embodiment, the subject is a mammal. When the subject is a mammal, the subject may be a human being.

The subject invention further provides a method for monitoring over a period of time the progression of an HIV infection in a subject infected with HIV which comprises: (a) determining the viral load of HIV in the subject according to the above-described method at a plurality of points suitably spaced over the period of time, thereby determining a plurality of viral loads; and (b) comparing the viral loads determined in step (a), thereby monitoring the progression of the HIV infection in the subject over the period of time.

The subject invention also provides a method for identifying whether a substance inhibits reverse transcriptase which comprises: (a) obtaining a sample comprising reverse transcriptase, the activity of the reverse transcriptase in the sample being predetermined; (b) contacting the sample with the substance; (c) determining the activity of reverse transcriptase in the sample resulting from step (b) according to the above-described method for determining in a sample the activity of an enzyme which catalyzes the synthesis of an RNA-DNA heteroduplex from a nucleic acid template; and (d) ascertaining whether the activity determined in step (c) is less than the predetermined activity of reverse transcriptase in the sample obtained in step (a), a lower activity in step (c) indicating inhibition of reverse transcriptase by the substance.

Samples comprising reverse transcriptase are well known and

readily obtainable by those of ordinary skill. Samples containing reverse transcriptase may be commercially obtained. Also, techniques for obtaining a sample of reverse transcriptase are well known and include, but are not limited to, purifying the lysates of transformed bacteria containing expression plasmids for reverse transcriptase.

The activity of reverse transcriptase in the sample obtained in step (a) may be predetermined by any known technique for determining reverse transcriptase activity. The reverse transcriptase activity of some commercially-obtainable samples is provided by the manufacturer. In one embodiment of the subject method, the activity of reverse transcriptase in the sample obtained in step (a) is predetermined according to the above-described method of the subject invention for determining in a sample the activity of an enzyme which catalyzes the synthesis of an RNA-DNA heteroduplex from a nucleic acid template.

Finally, this invention provides a kit for assaying an enzyme which catalyzes the synthesis of a double-stranded nucleic acid molecule from a nucleic acid template which comprises: (a) substrates for initiating catalysis by the enzyme; and (b) a suitable fluorophore capable of selectively binding to double-stranded DNA.

As used herein, assaying includes merely detecting an enzyme, as well as measuring the activity of an enzyme. The subject kit is useful in any of the methods described herein. For example, the subject kit is useful for detecting an HIV infection in a subject, for determining the viral load of HIV in a subject infected with HIV, and for identifying whether a substance inhibits reverse transcriptase.

This invention will be better understood from the Examples in the "Experimental Details" Section which follows. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of, and are not intended to, nor should they be construed to, limit the

invention as described more fully in the claims which follow thereafter.

Experimental Details

Materials and Methods

Materials

DAPI (4',6-diamidino-2-phenylindole) was purchased from Molecular Proves (Eugene, OR); Klenow fragment was obtained from Boerhinger-Mannheim (Indianapolis, IN); poly(A), oligo(dT) (n=10), and poly (dA)-oligo(dT) (n=10) were purchased from Pharmacia (Picscataway, NJ); [α-^{32}P]dTTP (3000 Ci/mmol) was purchased from NEN (Boston, MA); AZT (3'-azido-3'-deoxythymidine) 5'-triphosphate was purchased form Moravek Biochemicals (Brea, CA); nevirapine [6,11-dihydro-11-cyclopropyl-4-methyldipyrido [2,3-b:2',3'-e]-[1,4]diazepine-6-one] was obtained from Dr. Bruce Polsky (Infectious Disease Service, Memorial Sloan-Kettering Cancer Center (MSKCC)). Oltipraz was synthesized as described (8) by Dr. William G. Bornmann (Preparative Synthesis CORE Facility, MSKCC). All other chemicals and supplies were purchased from Fisher (Springfield, NJ) or Sigma (St. Louis, MO). Transformed bacteria containing expression plasmids for HIV-1 and HIV-2 RT were generously provided by Dr. Amnon Hizi (Sackler School of Medicine, Tel Aviv, Israel) (9). RT was purified to homogeneity utilizing a novel affinity purification approach (31). Aliquots of pooled active fractions from the intermediate purification steps were stored at −70°C, whereas active fractions from the final step were concentrated and stored at −70°C in 1:1 glycerol: RT buffer [50 mM Tris-Cl (pH 7.8), 75 mM KCl, 5mM $MgCl_2$, 1 mM DTT, 1mM EGTA, 0.1% Triton X-100, 0.1% NP-40, and 1 mg/ml bovine serum albumin (BSA)] without BSA. Crude and pure enzyme preparations were diluted (1:100-1:5000) into RT buffer shortly before use.

Isotopic RT Assay

The method of Flexner et al. (10) was used with slight modification. RT buffer was dispensed (9 μl/well) and mixed with (1 μl/well) diluted enzyme into a 96-well microtiter plate. The reaction was initiated with the addition of 25 μl/well RT buffer containing 10 μg/ml poly(A), 2.5 μg/ml oligo(dT), 5.6 μM dTTP,

and 20 μCi/ml [α-^{32}P]dTTP. After incubation for 30 min at 37°C, the plates were placed on ice and 20 μl aliquots were spotted onto DE-81 paper and air-dried. The paper was then washed three times in 2 X SSC (0.6 M NaCl, 0.06 M sodium citrate, pH 7.0) and dried, and the retained radioactivity was measured by scintillation counting.

Fluorometric Assay for RT Activity

The fluorometric assay for RT activity was derived from methods for the spectrofluorometric determination of DNA (11,12). RT diluted in RT buffer was dispensed into wells of a microtiter plate (40 μl/well), and nonenzymatic blanks were prepared by the addition of 40 μl/well of RT buffer without RT. The reaction was initiated by the addition of 100 μl/well of RT reaction buffer (RT buffer containing 20 μg/ml poly(A), 2 μg/ml oligo(dT), and 0-44.8 μM dTTP). After incubating the microtiter stopped by the addition of the 40 μl/well of 0.5 M EDTA (pH 7.8), and 150 μl aliquots of the resulting mixture were transferred to clear four-sided methacrylate cuvettes (1 X 1 X 4.5 cm) that contained 1 ml of 23 nM DAPI in 5 mM Tris·Cl, 8 mM NaCl (pH 7.8). The cuvettes were gently vortexed and incubated at room temperature in the dark for 30 min. The fluorescence was measured with a Perkin-Elmer 650S fluorescence detector ($\lambda_{excitation}$ = 359 nm; $\lambda_{emission}$ = 460 nm). Enzymatic activities were determined by subtracting the fluorescence of the nonenzymatic blanks from the fluorescence of the enzyme-containing cuvettes.

Fluorometric Assays with AZT 5'-Triphosphate or Nevirapine

In experiments with either AZT 5'-triphosphate or nevirapine, 10 μl of RT buffer was added to all wells of a 96-well plate. AZT 5'-triphosphate or nevirapine at concentrations of 1.12 μM or 1.12 mM, respectively, were added to the top row of microtiter wells in a volume of 10 μl. After thoroughly mixing the contents of the well, 10 μl/well was removed and mixed with the contents of the second row of wells. These twofold serial dilutions were performed with all but the last two rows of wells (no inhibitor control row and blank control row). RT buffer without RT was

then added to the last row of wells (30 µl/well), and the rest of the wells received 30 µl RT diluted into RT buffer. The reaction was initiated by the addition of 100 µl/well RT reaction buffer and was stopped at 30 min. The remainder of the assay procedure was carried out as described above. For K_i determinations, data was plotted according to the method of Dixon (13), and secondary transformations were generated according to the method of Segel (14), from which K_i values were determined. The IC_{50} results were determined from the x-intercept of the line calculated by the linear regression of data plotted according to the median-effect principle of Chou and Talalay (15).

Fluorometric Assay with Oltipraz as an Inactivator of RT

RT was preincubated with various concentrations of oltipraz as described (6). After incubation, 40 µl/well was transferred to a second microtiter plate and the remaining RT activity was assayed as described above. Data were analyzed according to the method described by Kitz and Wilson (16).

Assay of DNA Polymerase Activity of Klenow Fragment

The isotopic and fluorometric assay of DNA polymerase activity of Klenow fragment was assayed as described above for RT, except that the poly(A) and oligo(dT) were substituted with 20 µl/ml of poly(dA)·oligo(dT) in the RT reaction buffer.

Results and Discussion

DAPI becomes highly fluorescent upon binding to the minor groove of B-DNA (17,18) and is a useful reagent for quantitating the DNA content of biological specimens (11,12). The compound has preferential binding affinity for contiguous dA-dT base pairs, and the increase in fluorescence intensity is thought to result from prevention of an intramolecular proton transfer in the excited state that occurs (and quenches fluorescence) in solution (18). In preliminary experiments, we were able to demonstrate an increase in fluorescence after dTTP was incorporated into

poly(A)·oligo(dT) by RT, which led us to evaluate whether the fluorescence of DAPI could be used to measure RT activity.

Figure 1 shows the increase in DAPI fluorescence as a function of incubation time at several concentrations of RT. As is apparent, the assay is linear with incubation time and the amount of RT present in the assay (Fig. 1, inset). The time- and RT-dependent increase in fluorescence shown in Fig. 1 is completely abolished if: (a) RT is boiled for 5 min prior to dilution into RT buffer; (b) 40 µl/well of 0.5 M EDTA (pH 7.8) is added prior to the addition of RT reaction buffer; or (c) dTTP is omitted from the RT reaction buffer (data not shown). This indicates that the increase in fluorescence is an accurate measure of RT activity. This was confirmed by simultaneously measuring the incorporation of [α-^{32}P]dTTP into DNA and DAPI fluorescence with the same enzyme preparation. As can be seen in Fig. 2, the isotopic and fluorometric assays are exceedingly well-correlated to each other (r^2=0.986).

We assessed whether RT activity could be detected in crude preparations of RT by asking whether the increase in the specific activity of RT during the course of the purification of the enzyme could be determined with the fluorometric assay (Table 1). Although an increase in DAPI fluorescence was easily detected in lysates from transformed bacteria expressing HIV-1 RT (Table 1), bacterial lysates that do not express RT had no effect upon DAPI fluorescence (data not shown). The increase in specific activity of RT measures with the fluorometric assay correlated well with the increase in specific activity measured with the conventional isotopic assay (Table 1; r^2 = 0.988). This is also reflected by the relatively constant ratio of the isotopic to the fluorometric assay for each of the purification steps (Table 1, last column). Thus, the fluorometric assay can accurately measure RT activities from crude fractions, and we have found that the fluorometric assay can also detect the activity of RT during the chromatographic fractionation of the enzyme (data not shown). Although we routinely measure the fluorescence after 30 min incubation with DAPI, we have determined that the increase in fluorescence from crude or purified fractions is stable for up

to 24 h (Fig. 3).

The mean ± SE for the K_m of dTTP determined from Eadie-Hofstee plots with purified RT from four experiments was 7.71 ± 1.56 μM. This kinetic constant was easily determined with the fluorometric assay, was highly reproducible, and is within the range reported by other investigators (9, 19-21). Moreover, the assay can be utilized to characterize the type and magnitude of RT inhibition. This is demonstrated in Fig. 4 with three known inhibitors of HIV-1 RT. Although AZT 5'-triphosphate is a chain terminator of RNA directed DNA synthesis, its kinetic behavior is that of competitive inhibitor of RT with respect to dTTP. The K_i (11.1 ± 2.7 nM; N=2 experiments) determined with the fluorometric assay is similar to the K_i values published previously (22,23). The K_i value for nevirapine was determined to be 13.1 ± 6.3 μM (N=3 experiments), and is within the range of IC_{50} or K_i results published previously utilizing standard radiometric assays with dTTP as substrate (19,21,24). Although our Dixon plots appeared to indicate noncompetitive inhibition of RT with respect to dTTP (Fig. 4), secondary Segel transformations were consistent with mixed-type inhibition (Fig. 4, middle frame, inset). Mixed and/or noncompetitive inhibition has been observed with deoxynucleotide 5'-triphosphate substrates (24). The ability of oltipraz to inactivate RT activity was also evaluated by the fluorometric assay. As expected (6), addition of oltipraz to RT results in a time- and concentration-dependent loss of RT activity, which is kinetic evidence for the inactivation of the enzyme (16). Both K_i and k_3 (25.0 ± 3.5 μM and 0.085 ± 0.011 h^{-1}, respectively; N=2 experiments) were similar to previously published results utilizing an isotopic assay for RT activity (6).

We tested whether nevirapine and AZT 5'-triphosphate could inhibit the increased fluorescence of DAPI in assays conducted with HIV-2 RT. At 10 μM dTTP, the IC_{50} for nevirapine was 2.94 ± 0.28 μM (N=4 experiments) when HIV-1 RT was used as the enzyme source. As expected (20), nevirapine had no effect on the fluorometric RT assay when HIV-2 RT was assayed under identical conditions (<3% inhibition at 160 μM nevirapine). In stark

contrast, the IC_{50} for AZT 5'-triphosphate in the presence of 10 μM dTTP with HIV-2 RT as the enzyme source was 74.2 ± 8.4 nM (N=3 experiments). The IC_{50} for AZT 5'-triphosphate under identical conditions with HIV-1 RT was 15.2 ± 4.0 nM (N=3 experiments). Thus, our fluorometric assay can distinguish inhibitors that are specific for HIV-1 RT (e.g. nevirapine).

Our invention provides a new and novel fluorometric assay for RT activity. Our goal was to develop a rapid assay for RT activity so that we could more readily study the inactivation of this enzyme by 1,2-dithiole-3-thiones. Although we have not systematically determined whether there are buffers or reagents that interfere with the assay, we have demonstrated that RT activity can be accurately detected from crude samples that differ widely in ionic strength and composition (Table 1). We have determined that there is specificity for interaction between a polynucleotide duplex and a fluorescent probe with this assay. For example, we have established that the DNA polymerase activity of Klenow fragment cannot be detected by DAPI fluorescence when using poly(dA)·oligo(dT) as template·primer (data not shown; incorporation [α-^{32}P]dTTP into DNA was documented under our assay conditions). Thus, the presence of 2'-hydroxyl groups in the poly(A) strand of poly(A)·poly(dT) allows for DAPI binding (and perturbation in DAPI fluorescence). However, it is likely that another fluorescent probe could be found that would allow the fluorometric assessment of DNA polymerase activity with poly(dA)·oligo(dT). By development of suitable fluorometric assays, screening compounds for their ability to inhibit RT or DNA polymerase activity will become far easier to perform. Moreover, the assay which we have described may be used to measure RT activity in biological samples for laboratory and/or clinical studies.

TABLE 1

Comparison of RT Specific Activities Measured by the Conventional Isotopic RT Assay with the Fluorometric RT Assay

Purification Step	Isotopic Assay		Fluorometric Assay		
	RT spec act (nmol/min/mg)	Fold purification	RT spec act (F/mg)	Fold purification	Ratio of isotopic to fluorometric spec act
Bacterial lysate	2.8	≈1	4.2	≈1	0.67
$(NH_4)_2SO_4$ fraction					
Before Dialysis	17	6.1	30	7.2	0.57
After Dialysis	21	7.4	43	10	0.49
Gel filtration	64	23	98	24	0.65
Dye-ligand chromatography	430	150	860	210	0.50
MonoQ ion exchange	630	230	1500	370	0.42

Note. Activities were measured on pooled active fractions from a purification procedure of RT (see footnote 1) from transformed bacterial containing an expression plasmid for HIV-1 RT (9).

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What is claimed is:

1. A method for detecting in a sample an enzyme which catalyzes the synthesis of a double-stranded nucleic acid molecule from a nucleic acid template which comprises:
   (a) treating the sample under conditions sufficient to initiate catalysis by the enzyme;
   (b) contacting the sample with a suitable fluorophore which selectively binds to double-stranded DNA under conditions permitting said fluorophore to bind to double-stranded nucleic acid molecules in the sample synthesized by the catalysis; and
   (c) detecting the presence of fluorescence in the sample resulting from step (b), thereby detecting in the sample the enzyme.

2. The method of claim 1, further comprising treating the sample resulting from step (a) under conditions sufficient to terminate catalysis by the enzyme prior to contacting the sample with the fluorophore.

3. A method for determining in a sample the activity of an enzyme which catalyzes the synthesis of a double-stranded nucleic acid molecule from a nucleic acid template which comprises:
   (a) treating the sample under conditions sufficient to initiate catalysis by the enzyme;
   (b) contacting the sample with a fluorophore which selectively binds to double-stranded DNA under conditions permitting said fluorophore to bind to double-stranded nucleic acid molecules in the sample synthesized by the catalysis;
   (c) quantitatively measuring fluorescence in the sample resulting from step (b); and
   (d) calculating the activity of any enzyme in the sample as a predetermined function of the quantity of fluorescence measured in step (c).

4. The method of claim 3, further comprising treating the

sample resulting from step (a) under conditions sufficient to terminate catalysis by the enzyme prior to contacting the sample with the fluorophore.

5. A method for detecting an RNA-DNA heteroduplex in a sample which comprises:

(a) contacting the sample with a suitable fluorophore which selectively binds to double-stranded DNA under conditions permitting said fluorophore to bind to the RNA-DNA heteroduplex present in the sample; and

(b) detecting the presence of fluorescence in the sample resulting from step (a).

6. The method of claim 5, wherein the fluorophore is 4',6-diamidino-2-phenylindole; acridine orange; acridine homodimer; acridine-ethidium heterodimer; 9-amino-6-chloro-2-methoxyacridine; aminoactinomycin-D; a benzothiazolium-4-quinolinium dimer dye; a bisbenzamide dye; or ethidium homodimer.

7. The method of claim 6, wherein the fluorophore is 4',6-diamidino-2-phenylindole.

8. The method of claim 5, wherein the sample is a biological fluid.

9. The method of claim 5, wherein the sample is a tissue.

10. The method of claim 5, wherein the sample is obtained from a mammal.

11. The method of claim 10, wherein the mammal is a human.

12. The method of claim 5, further comprising quantitatively measuring fluorescence detected in step (b) so as to quantitatively determine the RNA-DNA heteroduplex in the sample.

13. A method for detecting in a sample an enzyme which catalyzes the synthesis of an RNA-DNA heteroduplex from a nucleic acid template which comprises:

(a) treating the sample under conditions sufficient to initiate catalyses of RNA-DNA heteroduplexes by the enzyme; and

(b) detecting the presence of RNA-DNA heteroduplexes in the sample resulting from step (a) according to the method of claim 5, thereby detecting in the sample the enzyme.

14. The method of claim 13, further comprising treating the sample resulting from step (a) under conditions sufficient to terminate catalysis by the enzyme prior to detecting the presence of RNA-DNA heteroduplexes in the sample.

15. The method of claim 13, wherein the enzyme is reverse transcriptase.

16. The method of claim 13, wherein the enzyme is RNA polymerase.

17. A method for determining in a sample the activity of an enzyme which catalyzes the synthesis of an RNA-DNA heteroduplex from a nucleic acid template which comprises:

(a) treating the sample under conditions sufficient to initiate catalyses of RNA-DNA heteroduplexes by the enzyme;

(b) quantitatively determining RNA-DNA heteroduplexes in the sample resulting from step (a) according to the method of claim 12; and

(c) calculating the activity of any enzyme in the sample as a predetermined function of the quantity of RNA-DNA heteroduplexes determined in step (b).

18. The method of claim 17, further comprising treating the sample resulting from step (a) under conditions sufficient to terminate catalysis by the enzyme prior to

quantitatively determining RNA-DNA heteroduplexes in the sample.

19. The method of claim 17, wherein the enzyme is reverse transcriptase.

20. The method of claim 17, wherein the enzyme is RNA polymerase.

21. A method for determining in a sample viral load of HIV which comprises:

(a) determining the activity of reverse transcriptase in the sample according to the method of claim 19; and

(b) calculating the viral load of HIV in the sample as a predetermined function of the activity of reverse transcriptase determined in step (a).

22. A method for diagnosing an HIV infection in a subject which comprises:

(a) obtaining a suitable sample from the subject; and

(b) detecting the presence of reverse transcriptase in the sample according to the method of claim 15, the presence of reverse transcriptase indicating an HIV infection.

23. A method for determining the viral load of HIV in a subject infected with HIV which comprises:

(a) obtaining a suitable sample from the subject; and

(b) determining the viral load of HIV in the sample according to the method of claim 21, thereby determining the viral load in the subject.

24. A method for monitoring over a period of time the progression of an HIV infection in a subject infected with HIV which comprises:

(a) determining the viral load of HIV in the subject according to the method of claim 23 at a plurality of points suitably spaced over the

period of time, thereby determining a plurality of viral loads; and

(b) comparing the viral loads determined in step (a), thereby monitoring the progression of the HIV infection in the subject over the period of time.

25. A method for identifying whether a substance inhibits reverse transcriptase which comprises:

(a) obtaining a sample comprising reverse transcriptase, the activity of the reverse transcriptase in the sample being predetermined;

(b) contacting the sample with the substance;

(c) determining the activity of reverse transcriptase in the sample resulting from step (b) according to the method of claim 19; and

(d) ascertaining whether the activity determined in step (c) is less than the predetermined activity of reverse transcriptase in the sample obtained in step (a), a lower activity in step (c) indicating inhibition of reverse transcriptase by the substance.

26. The method of claim 25, wherein the activity of reverse transcriptase in the sample obtained in step (a) is predetermined according to the method of claim 19.

27. A kit for assaying an enzyme which catalyzes the synthesis of a double-stranded nucleic acid molecule from a nucleic acid template which comprises:

(a) substrates for initiating catalysis by the enzyme; and

(b) a suitable fluorophore capable of selectively binding to double-stranded DNA.

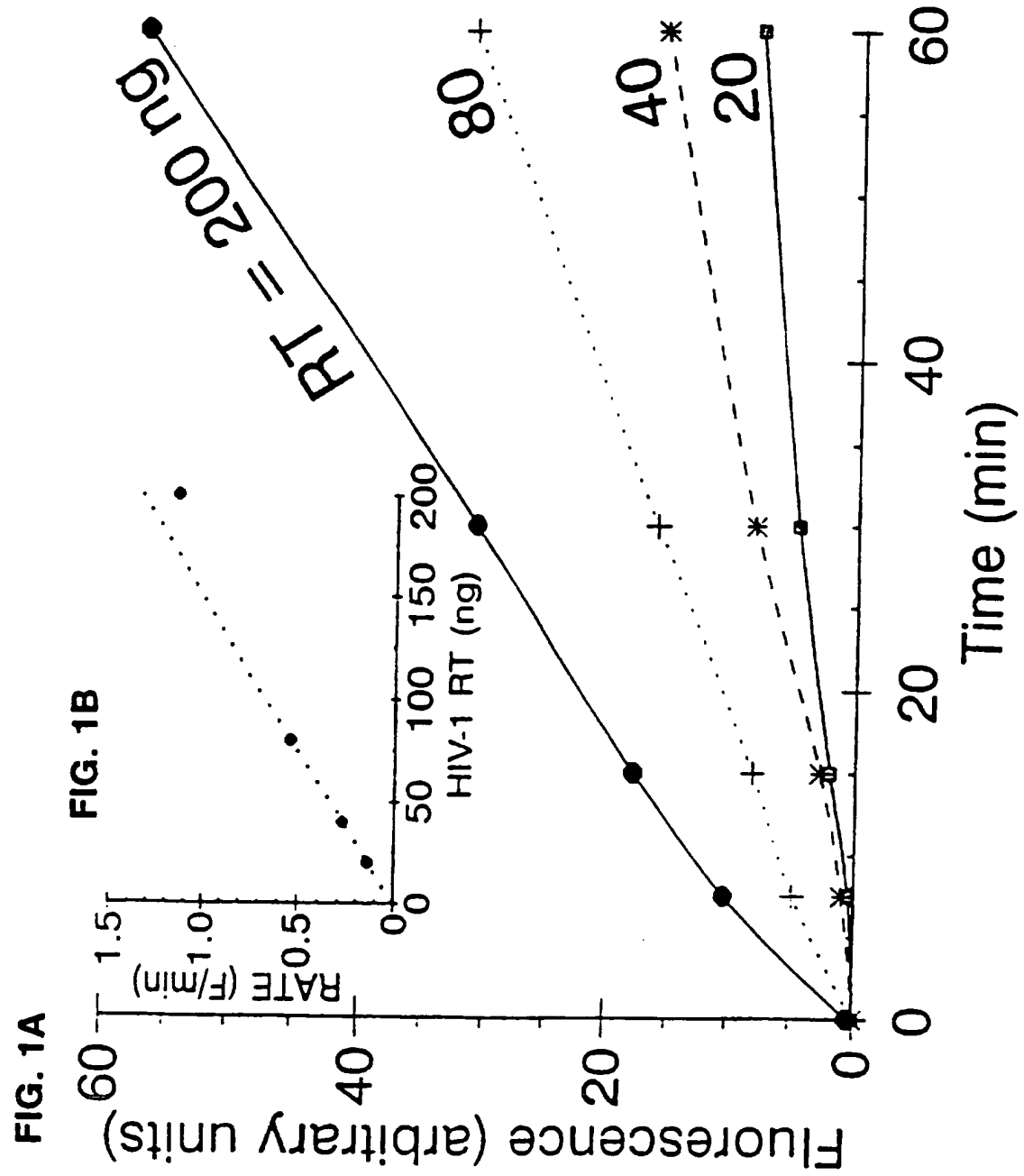

Fluorescence (arbitrary units)
Time (min)
0
20
40
60
80
RT = 200 ng
RATE (F/min)
HIV-1 RT (ng)
0
50
100
150
200
0.5
1.0
1.5

FIG. 1A

FIG. 1B

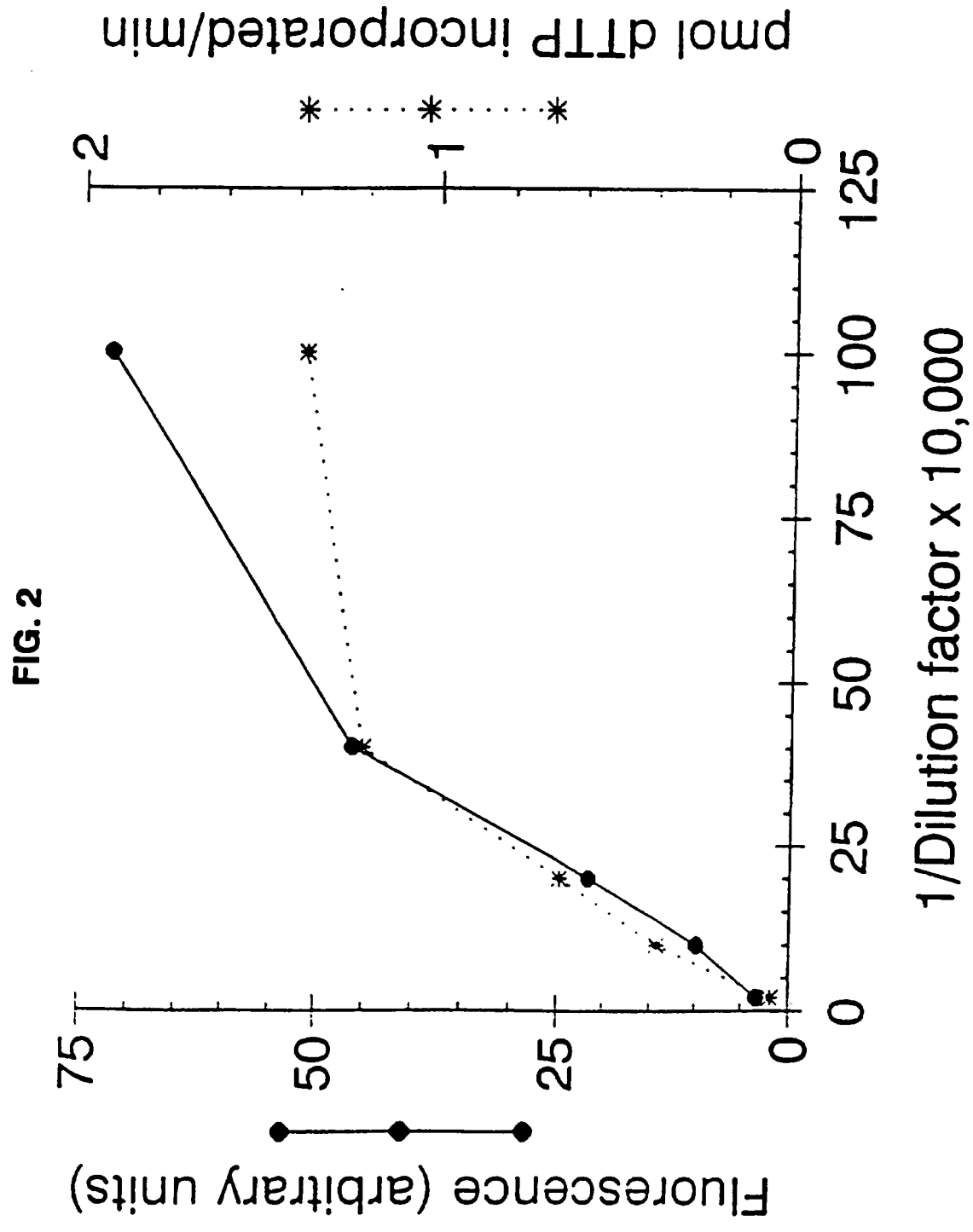
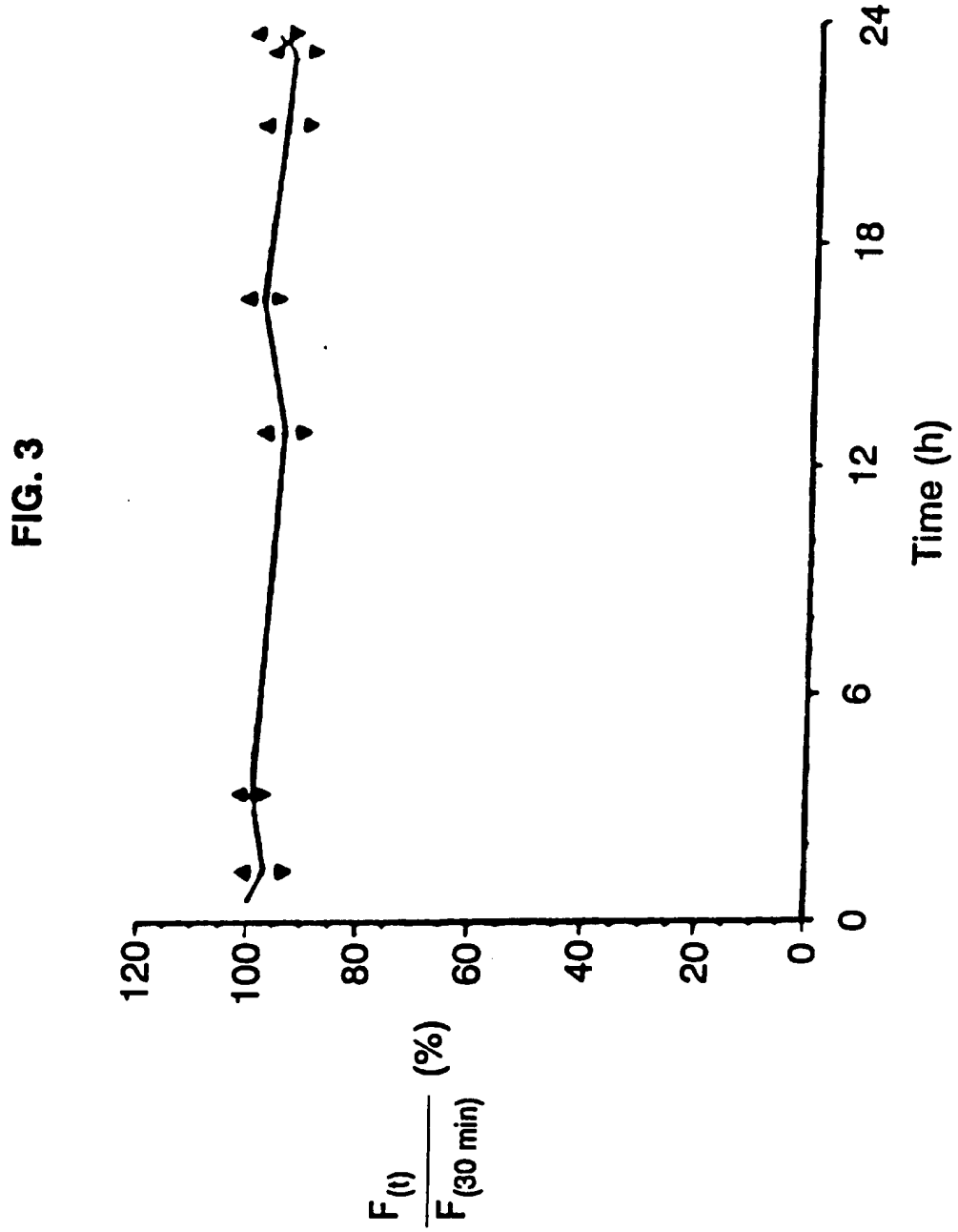

pmol dTTP incorporated/min
2
1
0
125
100
75
50
25
0
1/Dilution factor x 10,000
Fluorescence (arbitrary units)
75
50
25
0

FIG. 2

FIG. 3



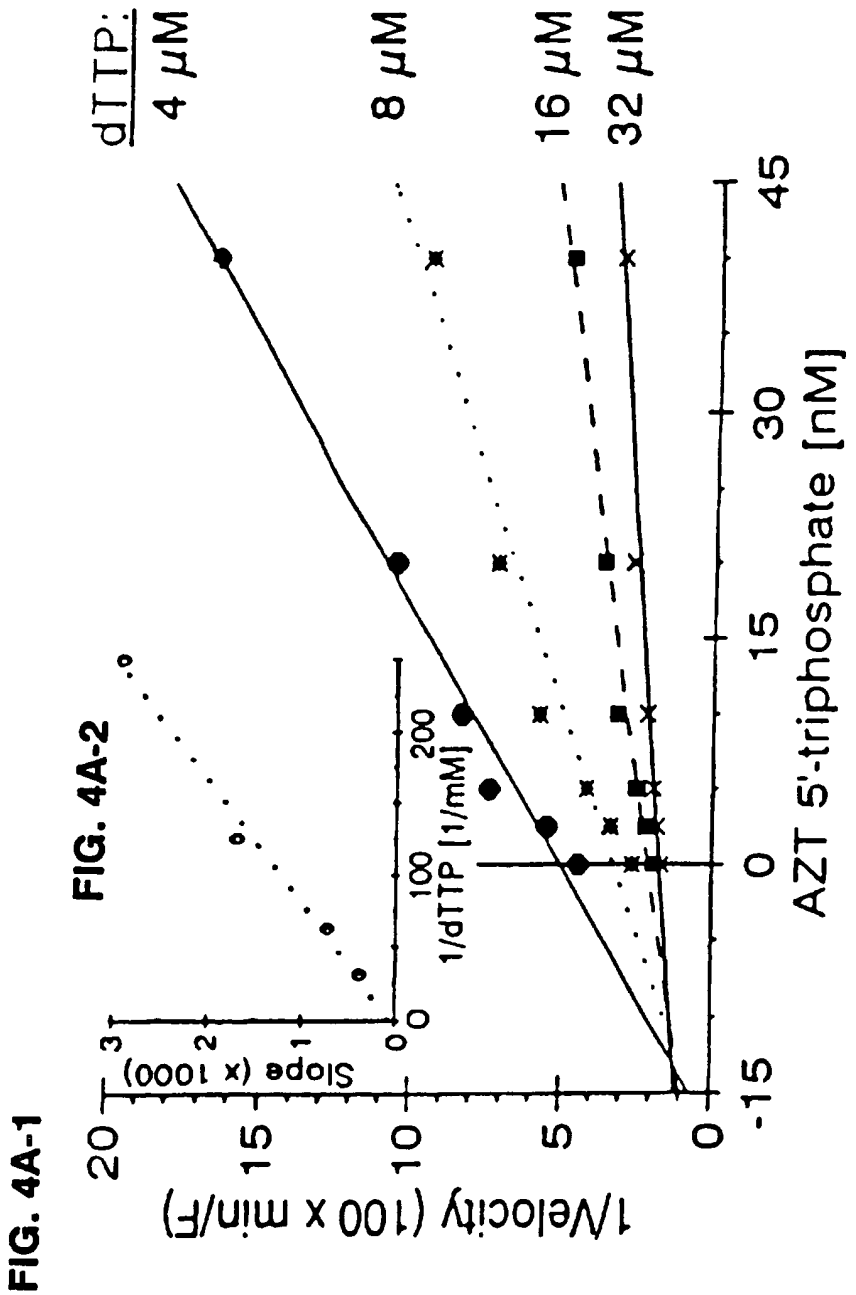


FIG. 4A-1

FIG. 4A-2

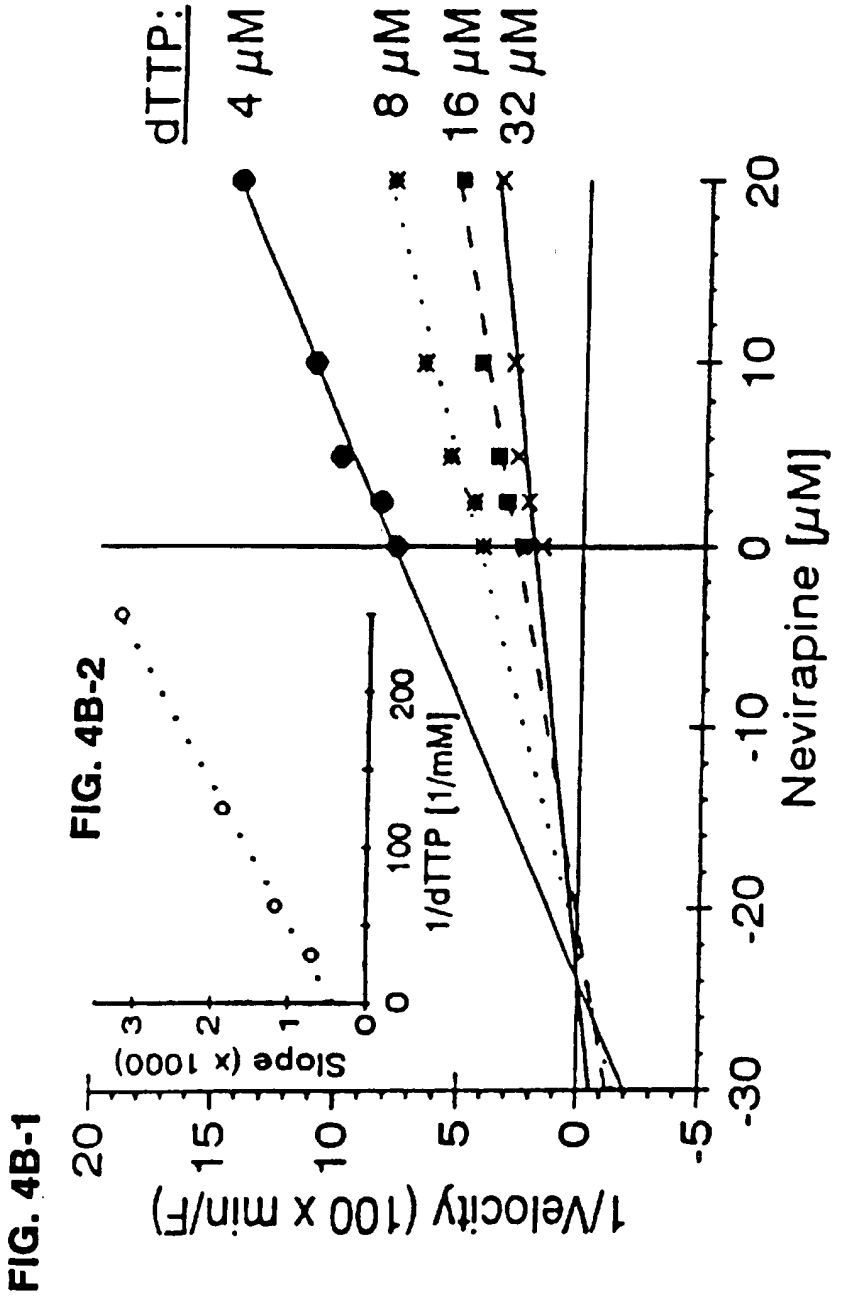
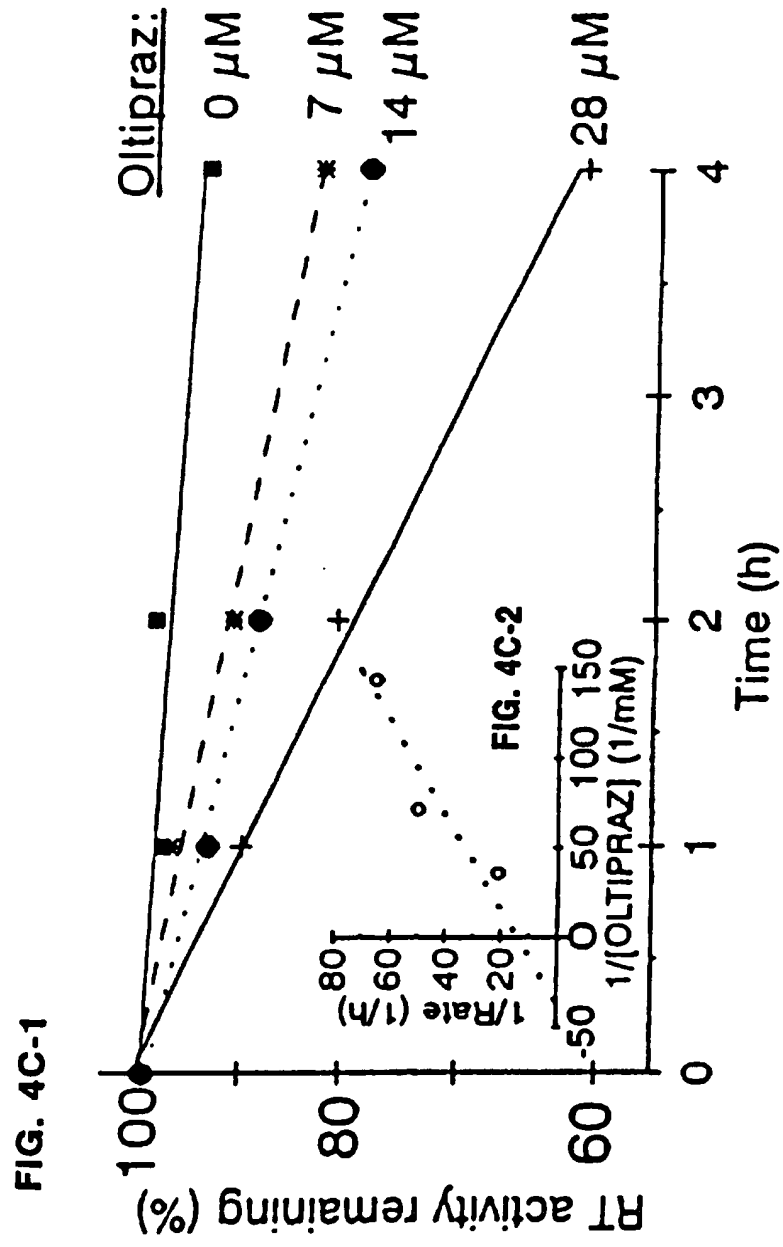


FIG. 4B-1

FIG. 4B-2


Oltipraz:
0 μM
7 μM
14 μM
28 μM
RT activity remaining (%)
100
80
60
0
1
2
3
4
Time (h)
1/Rate (1/h)
80
60
40
20
0
-50
50
100
150
1/[OLTIPRAZ] (1/mM)
FIG. 4C-1
FIG. 4C-2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/01836

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/70,1/68
US CL : 435/5, 6
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5, 6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAPLUS, BIOSIS, MEDLINE, WPIDS
search terms: reverse transcriptase, flurophore, DNA synthesis, RNA-DNA heteroduplex

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HEUVEL et al. Dioxin-responsive Genes: Examination of Dose-Response Relationships Using Quantitative Reverse Transcriptase-Polymerase Chain Reaction. Cancer Research 01 January 1994, Vol. 54, pages 62-67, see especially page 63, first column.	1-4 and 27
Y	The Promega Catalog, 1993-1994 Edition, page 64, see entire document.	1-4
Y	Perkin Elmer Cetus GeneAmp DNA Amplification Reagent Kit Product Insert, 17 October 1988, pages 1-2, see entire document.	27

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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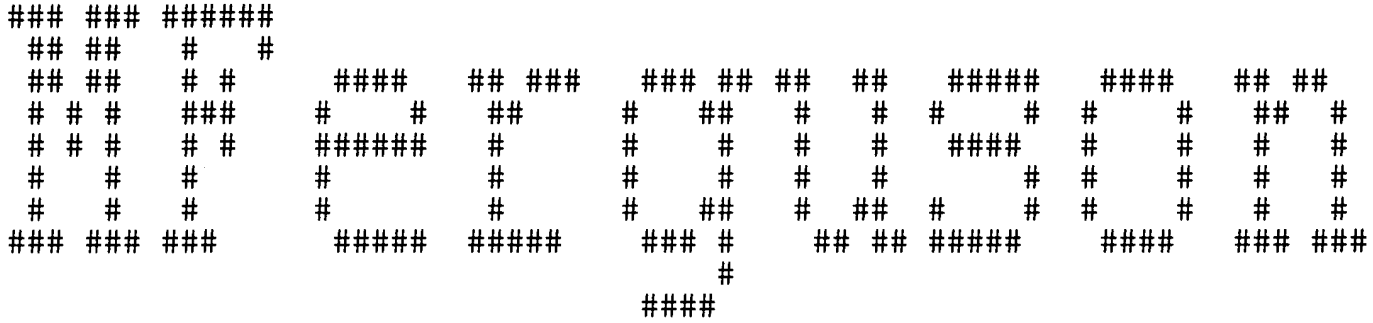
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HAUGLAND, R.P. 'Molecular Probes. Handbook of fluorescent probes and research chemicals,' published 1992 by Molecular Probes, Inc., Eugene, Oregon, pages 221-229, see entire document.	1-4 and 27



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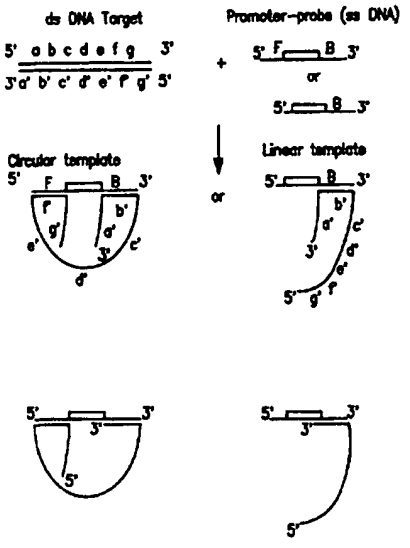

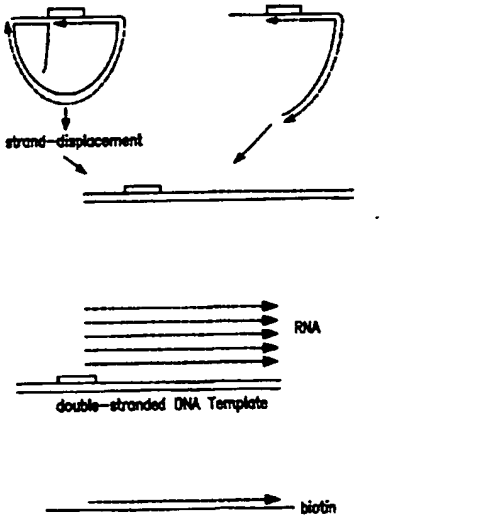

(57) Abstract

Continuous amplification reaction provides a method of amplifying a specific nucleic acid without the need to cycle a reaction. The method produces RNA transcripts which can be detected by a variety of methods. Amplification and detection kits are also provided.

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CONTINUOUS AMPLIFICATION REACTION

Cross-references to Related Application

This application is a continuation-in-part of U.S. Patent application Serial No. 07/792,585, filed on November 14, 1991.

Field of Invention

This invention relates to the field of nucleic acid amplification reactions in general and more particularly relates to a continuous amplification reaction, and reaction generating specific amplified RNA products from a DNA target.

Background of the Invention

The amplification and detection of specific nucleic acid sequences present in minute amounts is an increasingly important technique for identifying and classifying microorganisms, diagnosing infectious diseases, detecting and characterizing genetic abnormalities, identifying genetic changes associated with cancer, studying genetic susceptibility to disease, and measuring response to various types of treatment. Such procedures have also found expanding uses in detecting and quantitating microorganisms in foodstuffs, environmental samples, seed stocks, and other types of material where the presence of specific microorganisms may need to be monitored. Other applications are found in the forensic sciences, anthropology, archaeology, biology and clinical medicine where measurement of the relatedness of nucleic acid sequences has been used to identify criminal suspects, resolve paternity disputes, construct genealogical and phylogenetic trees, aid in classifying a variety of life forms, and identify disease states.

A common method for detecting and quantitating specific nucleic acid sequences is nucleic acid hybridization. The sensitivity of nucleic acid hybridization assays is limited primarily by the specific activity of the probe, the rate and extent of the

hybridization of the probe, and the sensitivity with which the label can be detected. The most sensitive procedures may lack many of the features required for routine clinical and environmental testing, such as speed, economy and convenience. Furthermore, their sensitivities may not be sufficient for many desired applications.

As a result of the interactions among the various components and component steps of this type of assay, there is often an inverse relationship between sensitivity and specificity. Thus, steps taken to increase the sensitivity of the assay (such as increasing the specific activity of the probe) may result in a higher percentage of false positive test results. The linkage between sensitivity and specificity has been a significant barrier to improving the sensitivity of hybridization assays. One solution to this problem would be to specifically increase the amount of target sequence present using an amplification procedure. Amplification of a unique portion of the target sequence without amplification of a significant portion of the information encoded in the remaining sequences of the sample could give an increase in sensitivity while at the same time not compromising specificity.

Amplification has been used to increase the sensitivity of nucleic acid assays. One common method for specifically amplifying nucleic acid sequences termed the "polymerase chain reaction" or "PCR" has been described by Mullis et al. (See U.S. patents 4,683,202 and 4,683,195 and European patent applications 86302298.4, 86302298.4, and 87300203.4 and *Methods in Enzymology*, Volume 155, 1987, pp. 335-350.) The procedure uses repeated cycles of primer dependent nucleic acid synthesis occurring simultaneously using each strand of a complementary sequence as a template. Therefore, at least two primers are required in PCR. The sequence amplified is defined by the primer molecules that initiate synthesis. The primers are complementary to the 3'-end portion of a target

sequence or its complement and must complex with those sites in order for nucleic acid synthesis to begin. After extension product synthesis, the strands are separated, generally by thermal denaturation, before the next synthesis step. In the PCR procedure, copies of both strands of a complementary sequence are synthesized.

The requirement of repeated cycling of reaction temperature between several different and extreme temperatures is a disadvantage of the PCR procedure.

The PCR procedure has been coupled to RNA transcription by incorporating a promoter sequence into one of the primers used in the PCR reaction and then, after amplification by the PCR procedure for several cycles, using the double-stranded DNA as template for the transcription of single-stranded RNA. (see e.g., Murakawa *et al.* DNA 7:827-295 (1988)). Other methods of amplifying nucleic acid sequences are also commercially available. These methods include the ligation amplification reaction (LCR), and the transcription-based amplification reaction. Ligation amplification reaction is described by Wu, D.Y and Wallace, R.B, *Genomics* 4:560-569 (1989) and Barringer, K.J., *et al.*, *Gene* 89:117-122 (1990). Transcription-based amplification reaction is described by Kwoh, D.Y., *et al.*, *Proc. Natl. Acad. Sci. USA* 86:1173-1177 (1989). These methods have the advantages of high sensitivity, but the disadvantages of being prone to false-positive results from reaction product contamination.

It is therefore an object of the present invention to amplify a target nucleic acid by continuous amplification reaction, which does not require repeated cycles of amplification and produces many RNA copies of the target sequence.

Another object of the present invention relates to detection of minute amounts of nucleic acids through use of a continuous amplification reaction (also referred to herein as "CAR").

Yet another object of the invention is to

indirectly amplify a target DNA signal by synthesizing and detecting multiple copy RNA molecules.

It is a further object of the present invention to provide a cost-effective, sensitive, solution hybridization assay for RNA transcripts produced by CAR.

Summary of the Invention

The present invention provides an amplification method, referred to herein as continuous amplification reaction ("CAR"). CAR is capable of producing detectable amounts of RNA transcripts from a minute amount of starting target region of a nucleic acid. This *in vitro* method for the enzymatic synthesis of RNA is based on an oligonucleotide primer containing a RNA polymerase promoter. This oligonucleotide is referred to herein as a promoter-primer.

The promoter portion of the promoter-primer may be flanked on either or both sides with regions homologous to one or two separate regions on the target nucleic acid molecule. Alternatively, the promoter-primer of the present invention may be a partially double-stranded oligonucleotide wherein it is double stranded within the promoter portion and single stranded within the primer portion of the oligonucleotide. A third alternative provides a promoter-primer which is a single oligonucleotide strand which is double stranded within the promoter region due to a stem loop formation in the oligonucleotide.

When the promoter is located in the center of the promoter-primer, hybridization between the promoter-primer and the target region of a nucleic acid forms a circular template-primer hybrid. Alternatively, the primer portion can be located downstream of the promoter on the promoter-primer in which case hybridization to the target region of a nucleic acid forms a linear structure.

Any nucleic acid may be amplified by the method of the present invention. A nucleic acid comprises a string of nucleotides of variable length. The nucleic

acid may be amplified in its entirety or a portion of the nucleic acid may be selected for amplification. In either case, the region of the nucleic acid containing the sequence(s) required for promoter primer hybridization and the sequence selected for transcription are referred to herein as the target region.

In the case where a portion of a nucleic acid is the target region, the 3' end of the nucleic acid may extend beyond the target region and such 3' flanking sequence is removed in a trimming step. This step may be accomplished by 3'-5' exonuclease digestion which may comprise a separate enzyme or the exonuclease activity associated with many nucleic acid polymerases. Alternatively, a unique restriction site may be created by hybridization of the denatured, single-stranded nucleic acid sequence to a trimming probe. The trimming probe comprises a sequence complementary to the 3' junction of the target region. The 3' junction of the target region comprises a sequence complementary to a portion of the target region and a sequence complementary to a region of the nucleic acid located 3' to the target region. Hence, restriction digestion of the site created by this hybrid molecule will generate a trimmed 3' end, wherein the product is ready for hybridization with a promoter-primer. Optionally, the trimming-probe may carry at least one ligand, capable of being captured on a solid matrix.

Under either of these conditions, the template-promoter primer hybrids are extended by the enzymatic activity of a nucleic acid polymerase. It may also be desirable to incorporate modified nucleotides into the 3' portion of the promoter-primer, such that the exonucleolytic activity associated with many nucleic acid polymerases will not digest any part of the promoter-primer. After polymerase extension along the length of template and promoter region, a double-stranded nucleic acid is formed. This product is subjected to transcription using, for example, RNA polymerase. In this

way, a template DNA can be indirectly amplified without the need to carry out any cycled reaction. Such transcripts can be detected by various methods including a hybrid-capture system.

In another aspect of the present invention, CAR provides an amplification reaction using a partially double-stranded promoter-primer which is double stranded in the promoter portion and single stranded in the primer portion. Prior to hybridization with the promoter primer, any 3' flanking sequence is removed. The removal or "trimming" of the 3' flanking sequence may be carried out with, for example a trimming probe, which removes the any single stranded sequences 3' of the target region. Upon hybridization of this promoter primer to target DNA, transcription is carried out. Optionally, a ligation reaction may be carried out to fill the gap between the promoter and the template. Further, it may be desired to produce a fully double stranded transcription template by first extending the partially double stranded hybrid with a nucleotide polymerase, preferably a DNA polymerase.

Kits are provided for screening samples for specific nucleic acid targets via CAR-produced RNA transcripts.

Brief Description of the Drawing

Figure 1 is a schematic diagram illustrating CAR. Step A shows the hybridization of a double stranded target DNA molecule with a single stranded promoter-primer. Step B shows the use of exonuclease, specific for 3'→5' cleavage which digests the excess single-stranded 3' ends of the DNA. Step C shows extension reactions using DNA polymerase, extending the 3' end of the promoter-probe and target DNA thereby producing a double-stranded DNA having a functional RNA polymerase promoter at its 5' end. Step D shows a transcription reaction with an RNA polymerase. Step E shows transcript hybridization to a DNA probe making an RNA/DNA hybrid, which provides one method of detecting CAR-produced transcripts.

Figure 2: Plasmid, illustrating the HIV-1 DNA region, with multiple internal restriction sites, used to generate CAR targets.

Figure 3: Schematic Drawing of the various combinations of hybrids useful in CAR.

Figure 4: Illustrative schematic of capture tail embodiment.

Figure 5: Ligase CAR with a partially double-stranded promoter primer and restriction site trimming probe.

Figure 6: Ligase CAR with loop promoter-primer and trimming probe.

Detailed Description of the Invention

The present invention relates to the amplification of a target nucleic acid. A method of amplification according to the present invention comprises denaturing a target nucleic acid forming a single-stranded nucleic acid strand; hybridizing said single-stranded nucleic acid to a promoter-primer forming a hybrid; trimming back the 3' end of the nucleic acid strand; extending the 3' end of promoter-primer and the trimmed 3' end of target strand with a nucleic acid polymerase forming a double-stranded nucleic acid having a functional RNA polymerase promoter; transcribing the double-stranded nucleic acid producing many RNA copies of the target sequence.

The ability to introduce a functional promoter to a specific site, in a target-dependent manner, allows the generation of at least 100 RNA transcripts from each specific nucleic acid target molecule. Coupling the CAR method with a specific and highly sensitive detection system, such as the Hybrid Capture system as described herein, permits the detection assay to be coupled with two levels of specificity. The first level of specificity is provided by targeting specific regions of a nucleic acid for amplification using the promoter-primer and the second level of specificity is achieved through use of a probe to

detect the newly transcribed RNA. The ability to indirectly amplify DNA target molecules via CAR inherently augments the level of detection of specific DNA sequences. The present invention provides the CAR approach, which allows the detection limits of specific nucleic acid sequences to be lowered.

Any source of nucleic acid, in purified or non-purified form, can be utilized as the test sample. For example, the test sample may be a food or agricultural product, or a human or veterinary clinical specimen. Typically, the test sample is a biological fluid such as urine, blood, plasma, serum, sputum or the like. Alternatively the test sample may be a tissue specimen suspected of carrying a nucleic acid of interest. The nucleic acid to be detected in the test sample is DNA or RNA, including messenger RNA, from any source, including bacteria, yeast, viruses, and the cells or tissues of higher organisms such as plants or animals. Methods for the extraction and/or purification of such nucleic acids have been described, for example, by Maniatis, et al., Molecular Cloning: A Laboratory Manual (New York, Cold Spring Harbor Laboratory, 1982).

The nucleic acid sequence to be detected in the test sample may be present initially as a discrete molecule so that the sequence to be detected constitutes the entire nucleic acid, or may only be a component of a larger molecule. It is not necessary that the nucleic acid sequence to be detected be present initially in a pure form. The test sample may contain a complex mixture of nucleic acids, of which the nucleic acid sequence to be detected may correspond to a gene of interest contained in total human genomic DNA, or a portion of the nucleic acid sequence of a pathogenic organism which organism is a minor component of a clinical sample.

The term "oligonucleotide" as the term is used herein refers to a nucleic acid molecule comprised of two or more deoxyribonucleotides or ribonucleotides. A

desired oligonucleotide may be prepared by any suitable method, such as purification from a naturally occurring nucleic acid, or de novo synthesis. Examples of oligonucleotides are probes and promoter-primers described herein.

The term "RNA transcript" as the term is used herein refers to a ribonucleic acid molecule synthesized by an RNA polymerase enzyme under the control of the promoter-primer. The RNA transcript of a specific nucleic acid sequence is either homologous or complimentary to that sequence.

Continuous Amplification Reaction ("CAR") is capable of amplifying a nucleic acid template in order to produce a detectable amount of RNA product. The amplification method can detect as little as 10-100 molecules of nucleic acid. The method uses an oligonucleotide comprising at least one segment complementary to one strand of a target sequence and a segment containing a promoter. This oligonucleotide primer, when hybridized to a strand of a template, preferably the anti-sense strand, and extended can generate a copy of the target nucleic acid with the capability of transcription via the added promoter sequence. The promoter is added to the 5′ end of the strand to be transcribed. If the anti-sense strand of a target region is used for the hybridization step, the promoter is added to the 5′ end of the coding strand of the target nucleic acid.

In one preferred embodiment, a nucleic acid and a promoter-primer are hybridized. The primer portion of the promoter-primer is designed to be complementary to non-contiguous portions of the target region. For example portions at both ends of the target region of the nucleic acid may be selected for hybridization. In addition, the promoter-primer is designed to contain a promoter sequence for an RNA polymerase. Upon hybridization, the primer portions of the promoter-primer link the 5′ and 3′ end

portions of the target region of nucleic acid, such that the promoter sequence portion is sandwiched between the two hybridized end sequences. The result of this hybridization is the formation of a circle. Hereinafter, this embodiment is referred to as "circular CAR". In the case where the target region is a segment of a larger nucleic acid hybridization of the promoter-primer results in the formation of a circle with at least one dangling end.

Any single stranded 3' sequence flanking the target region may then be trimmed back to produce a 3' end of the target sequence which is flushed with the hybridized promoter-primer. "Flushed" as the term is used herein refers to a double-stranded end with no single stranded sequence at the end of the target nucleic acid: For example, a 3' flushed sequence may be produced by the triming step. Simultaneous with or subsequent to the trimming back step is the extension step which extends the 3' ends of the hybrid structure via a nucleic acid polymerase forming an extension product. The nucleic acid polymerase extends the 3' end of the promoter-primer along the template to form a double stranded intermediate. The polymerase also extends the trimmed 3' end of the template thereby extending the nucleic acid so that the resulting intermediate product will be double-stranded along its entire length and carry a functional transcriptional promoter. Finally, the double-stranded extension product is transcribed by an RNA polymerase, generating multiple RNA transcripts from each extension product. The promoter, originally part of the promoter-primer, facilitates the action of the RNA polymerase, resulting in the production of many RNA transcripts from the copied target nucleic acid.

Another embodiment of the present invention relates to the use of an oligonucleotide promoter primer, wherein the primer portion is complementary to a 3' portion of the target region in a nucleic acid sequence.

This embodiment is hereinafter referred to as "linear CAR". The promoter portion of the promoter-primer is located at the 5' end. After hybridization is complete, 3' single stranded sequences flanking the target region may be trimmed back to produce a 3' flushed end. Simultaneous with or subsequent to removal of the single stranded 3' flanking sequence, extension is carried out via the activity of a nucleic acid polymerase, producing an extension product carrying the newly added promoter region. The resulting double stranded nucleic acid is then transcribed with an RNA polymerase, facilitated by the newly added promoter sequence.

Yet another embodiment of the present invention, which is referred to as double-stranded-CAR ("ds-CAR"), relates to the use of a partially double-stranded promoter-primer (referred to as "ds-promoter-primer"), wherein the promoter-primer is double-stranded within the promoter portion and is single-stranded within a region downstream to the promoter which is complementary to the 3' end of the target region.

In one embodiment of ds-CAR, upon hybridization, the 3' end of the DNA target directly abuts the 5' end of the short strand of the ds-promoter-primer. This hybrid structure may be directly subjected to transcription. Optionally, ligase may then be reacted with this hybrid forming a continuous, partially double-stranded template which is also transcription-ready. Yet another optional step may include extending either of the above described partially double stranded molecules (either ligated or non-ligated) with a DNA polymerase, thereby producing a fully double stranded template, also ready for transcription (see, for example, Zhou, et al. 1995 *Cell* 82, 577-585). Transcription is then carried out using an RNA polymerase to produce many RNA transcripts.

The partially double stranded promoter-primer of the present invention may be made up of multiple oligonucleotides, which have been hybridized or covalently

linked. Alternatively the ds-promoter-primer may be a single oligonucleotide strand with intramolecular folding capabilities such that a stem-loop structure is formed, wherein the stem is formed within the promoter portion while the primer portion remains single stranded. In either case the double stranded portion of the promoter-primer comprises a RNA polymerase promoter sequence, whereas the single stranded portion comprises a sequence complementary to a 3' portion of the target region of a nucleic acid.

In another embodiment of CAR, the single stranded (i.e. denatured) target DNA may be hybridized to a complementary oligonucleotide which is fixed to a solid matrix (see fig. 4). This step may serve to separate the nucleic acid of interest from other molecules in a sample. If the target sequence has been hybridized to the immobilized oligonucleotide by its 5' flanking sequence, the immobilized target may then be hybridized with the promoter-primer of the present invention and the steps of amplification may be carried out as described above and herein below.

Denaturing a sample may be necessary to carry out the assay of the present invention in cases where the target nucleic acid is found in a double-stranded form or has a propensity to maintain a rigid structure. Denaturing is a step producing a single stranded nucleic acid and can be accomplished by several methods well-known in the art (Sambrook et al. (1989) in "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Press, Plainview, New York). One preferred method for denaturation may be heat, for example 90-100°C, for about 2-20 minutes.

Alternatively, a base may be used as a denaturant when the nucleic acid is a DNA. Many known basic solutions are useful for denaturation, which are well-known in the art. One preferred method uses a base, such as NaOH, for example, at a concentration of 0.1 to 2.0 N NaOH at a temperature of 20-100°C, which is

incubated for 5-120 minutes. Treatment with a base, such as sodium hydroxide not only reduces the viscosity of the sample, which in itself increases the kinetics of subsequent enzymatic reactions, but also aids in homogenizing the sample and reducing background by destroying any existing DNA-RNA or RNA-RNA hybrids in the sample.

The target nucleic acid molecules are hybridized to a promoter-primer complementary to the target region of a nucleic acid. Hybridization is conducted under standard hybridization conditions well known to those skilled in the art. Reaction conditions for hybridization of an oligonucleotide promoter-primer to a nucleic acid sequence vary from oligonucleotide to oligonucleotide, depending on factors such as oligonucleotide length, the number of G and C nucleotides, and the composition of the buffer utilized in the hybridization reaction. Moderately stringent hybridization conditions are generally understood by those skilled in the art as conditions approximately 25°C below the melting temperature of a perfectly base-paired double stranded DNA. Higher specificity is generally achieved by employing incubation conditions having higher temperatures, in other words more stringent conditions. Chapter 11 of the well-known laboratory manual of Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, second edition, Cold Spring Harbor Laboratory Press, New York (1990) (which is incorporated by reference herein), describes hybridization conditions for oligonucleotide probes and primers in great detail, including a description of the factors involved and the level of stringency necessary to guarantee hybridization with specificity.

The promoter-primers and target nucleic acids are incubated for approximately 5 to 120 minutes at about 20 to 80°C to allow hybridization. Preferably, promoter-primer and target nucleic acid are incubated for about 20 to 60 minutes at about 30 to 70°C. Most preferably, the

promoter-primer and target nucleic acid in the sample are incubated for about 30 minutes at about 35-50°C.

Hybridization is typically performed in a buffered aqueous solution, for which the conditions of temperature, salts concentration, and pH are selected to provide sufficient stringency such that the promoter-primer will hybridize specifically to the target nucleic acid sequence but not any other sequence. Generally, the efficiency of hybridization between promoter-primer and target will be improved under conditions where the amount of promoter-primer added is in molar excess to the template, preferably a 1000 to 10^6 molar excess. It is understood, however, that the amount of target nucleic acid in the test sample may not be known, so that the amount of promoter-primer relative to template cannot be determined with certainty.

Alternatively, if the target DNA has been treated with base, the promoter-primer is diluted in a probe diluent that also acts as a neutralizing hybridization buffer. In this manner, the pH of the sample can be kept between pH 6 and pH 9, which will favor the hybridization reaction and will not interfere with subsequent enzymatic reactions. Preferably, the neutralizing buffer is a 2-[bis(2-hydroxyethyl) amino] ethane sulfonic acid ("BES") (Sigma, St. Louis, MO) and sodium acetate buffer. Most preferably, the neutralizing hybridization buffer is a mixture of 2 M BES, 1 M sodium acetate, 0.05% of an antimicrobial agent, such as NaN_3, 5 mM of a chelating agent, such as EDTA, 0.4% of a detergent, such as Tween-20™ and 20% of a hybridization accelerator, such as dextran sulfate. The pH of the neutralizing hybridization buffer is between approximately 5 to 5.5.

The promoter-primer of the present invention comprises a promoter portion and a primer portion. The primer portion will vary in sequence depending upon the target sequence. The primer portion comprises a length of

at least 8 bases and may be as long as desired, for example to maximize specificity of hybridization. The promoter portion may comprise any RNA polymerase promoter sequence known in the art such as those described by Chamberlin and Ryan (1982 In: *The Enzymes*. San Diego, CA, Academic Press: 15:87-108) and Jorgensen, et al (1991 *J. Biol. Chem.* 266:645-655). Several RNA polymerase promoter sequences are preferred: these include but are not limited to promoters derived from SP6 (Zhou & Doetsch, 1993 *Proc. Natl. Acad. Sci. USA* 90:6601-6605), T7 (Martin & Coleman, 1987 *Biochemistry*, 26:2690-2696) and T3 (McGraw, et al., 1985 *Nucl. Acid. Res.*, 13:6753-6766). Preferred is an RNA promoter sequence derived from *Thermus thermophilus* (Wendt et al. 1990 *Eur. J. Biochem.*, 191:467-472; Faraldo et al. 1992 *J. Bact.*, 174:7458-62; Hartmann et al. 1987 *Biochem*, 69:1097-1104, Hartmann et al. 1991 *Nucl. Acids Res.* 19:5957-5964). The length of the promoter portion of the promoter-primer will vary depending upon the promoter sequence chosen. For example, the T7 RNA polymerase promoter may be as short as 25 bases in length to act as functional promoters, while other promoter sequences require 50 or more bases to provide a functional promoter.

The promoter-primer may be produced by any suitable method known in the art, including by chemical synthesis, isolation from a naturally-occurring source, recombinant production and asymmetric PCR (McCabe, 1990 In: *PCR Protocols: A guide to methods and applications*. San Diego, CA., Academic Press, 76-83). It may be preferred to chemically synthesize the promoter-primer in one or more segments and subsequently link the segments. Several chemical synthesis methods are described by Narang et al. (1979 *Meth. Enzymol.* 68:90), Brown et al. (1979 *Meth. Enzymol.* 68:109) and Caruthers et al. (1985 *Meth. Enzymol.* 154:287), which are incorporated herein by reference. Alternatively, cloning methods may provide a convenient nucleic acid fragment which can be isolated for

use as a promoter primer. The overall nucleic acid composition of the promoter-primer will vary depending upon the target nucleic acid chosen and the type of CAR employed. The length of the promoter-primer will also vary depending upon the target nucleic acid, the promoter chosen and the degree of hybridization specificity desired.

In producing the promoter-primer of the present invention it may be desirable to modify the nucleotides or phosphodiester linkages in one or more positions of the promoter primer. For example, it may be advantageous to modify at least the 3' portion of the promoter-primer. Such a modification prevents the exonuclease activity from digesting any portion of the promoter-primer. It is preferred that at least the ultimate and penultimate nucleotides or phosphodiester linkages be modified. One such modification comprises a phosphorothioate compound which, once incorporated inhibits 3' exonucleolytic activity on the promoter-primer. It will be understood by those skilled in the art that other modifications of the promoter-primer, capable of blocking the exonuclease activity can be used to achieve the desired enzyme inhibition.

The trimming step of the present invention may be carried out by various means. The most common method of trimming back 3' ends utilizes the enzymatic activity of exonucleases. In particular, specific directional exonucleases facilitate a 3'-5' trimming back of the target DNA-promoter primer hybrid. Such exonucleases are known within the art and include, but are not limited to, exonuclease I, exonuclease III and exonuclease VII. Preferred, however, is the 3'-5' exonuclease activity associated with many nucleic acid polymerases. Using such nucleic acid polymerases reduces the number of enzymes required in the reaction and provides the appropriate activity to trim back the free 3' flanking ends of the target DNA.

Alternatively, it may be preferred to use a trimming probe method. A trimming probe may be particularly useful in cases where a long single-stranded sequence 3' to the target region is generated upon hybridization of a nucleic acid with a promoter-primer. The trimming probe technique is carried out prior to hybridization with a promoter-primer.

A trimming probe comprises a single stranded oligonucleotide which contains sequence complementary to a 3' portion of the target region of the nucleic acid. The 3' junction further comprises a potential restriction endonuclease recognition site, but for the fact that it is only present as a single strand. Restriction endonucleases are enzymes which specifically recognize and cleave a nucleic acid sequence. The restriction endonuclease recognition sequences vary in length but require a double-stranded sequence. These recognition sites are well-known in the art. Similarly restriction endonucleases are numerous and are well-known in the art. Sambrook et al. (1990 Molecular Cloning: A Laboratory Manual, second edition, Cold Spring Harbor Press, N.Y.) provides a review of many restriction endonucleases and their recognition sequences and is incorporated herein by reference.

The trimming probe procedure requires hybridization of the single stranded target sequence to the trimming probe. Hybridization creates a short double-stranded region forming a functional restriction endonuclease recognition sequence. The restriction endonuclease is then able to digest the target sequence as defined by the restriction cleavage site. Preferably, this restriction digestion reaction produces a target region having a flushed 3' end.

Optionally, the trimming probe may also carry a ligand in one or more positions, capable of being captured onto a solid matrix. A ligand conjugated-trimming probe provides a convenient way of separating the target DNA

from other molecules present in a clinical sample. Once the ligand conjugated-capture probe - target sequence hybrid is trapped on a solid matrix via the ligand, the solid matrix is washed thereby separating the hybrid from all other components in the sample. The washed immobilized hybrid is subjected to restriction endonuclease digestion. After digestion, the target sequence is released from the solid matrix while the 5' end of the probe remains immobilized on the solid matrix. A small denaturation step allows the remaining portions of the trimming probe to be removed and the target sequence can then be hybridized to a promoter-primer molecule for amplification.

Use of a trimming probe is particularly advantageous in ligase CAR in the situation where a 5' sequence flanks the target region.

Many known ligands may be used in the trimming probe, including vitamin derivatives antigen-antibody complexes, metal derivatives and the like. In one preferred embodiment, biotin is used as the ligand, wherein the trimming probe is tagged with biotin and the solid matrix is coated with a strong binding molecule, such as avidin, streptavidin, or their derivatives. Various combinations of ligand and ligand-binding agent are well known and may be used to capture the hybrid onto a solid matrix. For example, digoxigenin and anti-digoxigenin 2, 4-dinitrophenol (DNP) and anti-DNP may be used. Fluorogens, such as fluorescein, phycoerythrin, allo-phycocyanin, phycocyanin rhodamine, Texas red or other proprietary fluorogens may be used in combination with an anti-fluorogen specific antibody.

Solid matrices useful in capturing the ligand-conjugated probe are available to the skilled artisan. Solid phases useful to serve as a matrix for the present invention include but are not limited to polystyrene, polyethylene, polypropylene, polycarbonate or any solid plastic material in the shape of test tubes, beads

microparticles, dip-sticks or the like. Additionally, matrices include, but are not limited to membranes, 96-well microtiter plates, test tubes and Eppendorf tubes. Solid phases also include glass beads, glass test tubes and any other appropriate shape made of glass. A functionalized solid phase such as plastic or glass which has been modified so that the surface carries carboxyl, amino, hydrazide or aldehyde groups can also be used. In general such matrices comprise any surface wherein a ligand-binding agent can be attached or a surface which itself provides a ligand attachment site.

The single stranded target nucleic acid is hybridized to a promoter-primer. Trimming, as described above is either carried out prior to or simultaneous with the extension step.

"Extension" as the term is used herein is the addition of nucleotides to the 3' hydroxyl end of a nucleic acid wherein the addition is directed by the nucleic acid sequence of a template. Most often the extension step is facilitated by an enzyme capable of synthesizing DNA from an oligonucleotide primer and a template. Suitable enzymes for these purposes include, but are not limited to, for example, *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase I, T4 DNA polymerase, Vent™ (exonuclease plus) DNA polymerase, Vent™ (exonuclease minus) DNA polymerase, Deep Vent™ (exonuclease plus) DNA polymerase, Deep Vent™ (exonuclease minus) DNA polymerase, 9°N_m DNA polymerase (New England BioLabs), T7 DNA polymerase, Taq DNA polymerase, Tfi DNA polymerase (Epicentre Technologies), Tth DNA polymerase, Replitherm™ thermostable DNA polymerase and reverse transcriptase. One or more of these agent may be used in the extension step of CAR. To maximize efficiency, it may be desirable to use one agent for both the extension and trimming steps. Additional reagents may be added as necessary to shift the kinetic parameters of the polymerase enzyme to either increase or

decrease its extension rate and/or 3'-5' and 5'-3' exonucleolytic activity. The extension step produces a double-stranded nucleic acid having a functional promoter at its 5' end.

Once formed, the double-stranded extension product serves as a template for RNA transcript production.

Transcription of the double-stranded extension product or partially double stranded ligation product carrying a functional promoter sequence is facilitated by an RNA polymerase. Many such polymerases are known in the art, including, but not limited to SP6 RNA Polymerase, T7 RNA polymerase and T3 RNA polymerase. A preferred RNA polymerase is *Thermus thermophilus* derived RNA polymerase. One or more such RNA polymerases may be employed in the transcription step of the CAR method.

Under suitable reaction conditions, including the presence of the necessary reagents, the synthesis of RNA transcripts will occur continuously and in proportion to the amount of the nucleic acid sequence to be detected that was originally present in the test sample. Additional reagents may be added as necessary to prepare the desired quantity of RNA transcripts. These reagents may be used to shift the equilibrium of the transcription reaction to either increase or decrease the transcription rate and efficiency as desired. One such reagent, inorganic pyrophosphatase, may be used to increase transcription yields and minimize the effect of magnesium ion concentration in the transcription reaction (Cunningham & Ofengand, 1990 *BioTechniques*, 9:713-714). Preferably the synthesis of RNA transcripts will be carried out in the presence of a ribonuclease inhibitor, as for example vanadyl-ribonucleoside complexes or human placental ribonuclease inhibitor, in order to avoid possible degradation of the transcripts by any adventitious ribonuclease contaminant. (Berger, 1987, *Meth. Enzymol.*, 152:227; de Martynoff et al., 1990,

Biochem. Biophys. Res. Commun. 93:645; Sheel et al., 1979, *Proc. Natl. Acad. Sci. USA* 76:4898). After the appropriate length of time has passed to produce the desired quantity of RNA transcripts, the reaction may be halted by inactivating the RNA polymerase in any known manner or separating the components of the reaction.

The amplification reaction of the present invention produces transcripts which may be detected using various methods. For example, the transcripts may be directly detectable by addition of a labeled nucleotide in the transcription reaction. In many situations, it may be preferred to use label dUTP, since this nucleotide is specific to RNA molecules and hence its incorporation will be limited to transcription reaction products.

Many different labels may be used in generating detectable transcripts. Preferred methods of labeling RNA transcripts are with ^{32}P or ^{35}S using RNA polymerases. In addition, there are known non-radioactive techniques for signal amplification including methods for attaching chemical moieties to pyrimidine and purine rings (Dale, R.N.K. et al. (1973) *Proc. Natl. Acad. Sci. USA*, 70:2238-2242; Heck, R.F. (1968) *S. Am. Chem. Soc.*, 90:5518-5523), methods which allow detection by chemiluminescence (Barton, S.K. et al. (1992) *J. Am. Chem. Soc.*, 114:8736-8740) and methods utilizing biotinylated nucleic acid probes (Johnson, T.K. et al. (1983) *Anal. Biochem.*, 133:125-131; Erickson, P.F. et al. (1982) *J. of Immunology Methods*, 51:241-249; Matthaei, F.S. et al (1986) *Anal. Biochem.*, 157:123-128) and methods which allow detection by fluorescence using commercially available products.

Alternatively, nucleic acid probes may be used to detect CAR-produced RNA transcripts. Nucleic acid probes are detectable nucleic acid sequences that hybridize to complementary RNA or DNA sequences in a test sample. Detection of the probe indicates the presence of a particular nucleic acid sequence in the test sample for which the probe is specific. In addition to aiding

scientific research, DNA or RNA probes can be used to detect the presence of viruses and microorganisms such as bacteria, yeast and protozoa as well as genetic mutations linked to specific disorders in patient samples. Grunstein *et al., Proc. Natl. Acad. Sci. USA* 72:3961 (1975) and Southern, *J. Mod. Biol.* 98:503 (1975) describe hybridization techniques using radiolabelled nucleic acid probes. Nucleic acid hybridization probes have the advantages of high sensitivity and specificity over other detection methods and do not require a viable organism. Hybridization probes are often labelled with a substance that can be easily detected. For example, a radioactive hybridization assay for human papillomavirus (HPV) is currently commercially available as a Profile™ kit from Digene Diagnostics (Silver Spring, MD).

Hybridization can also be detected with the aid of an antibody specific for a labelled probe as described in U.S. Patent No. 4,743,535 to Carrico. The probe is labelled with a detectable substance such as flavin adenine dinucleotide (FAD) or a fluorescent agent. An antibody specific for the labelled probe, after it has hybridized to the sample nucleic acid, is detected by a biochemical reaction.

Unlabeled transcripts may also be detected in the present invention. These transcripts may be detected by many techniques known in the art. For example, hybridization assays for the detection of RNA have been developed. For example, a hybridization protection assay for RNA is commercially available from Gen-Probe Inc. (San Diego, CA). The hybridization protection assay employs a single-stranded nucleic acid probe linked to an acridinium ester, as described by Engleberg, N.C., *ASM News* 57:183-186 (1991), Arnold et al. *Clin. Chem.* 35:1588-1594 (1989) and U.S. Patent No. 4,851,330. Hybridization of the probe to a target RNA molecule protects the acridinium ester bond from base hydrolysis so that the detected chemiluminescent signal is proportional to the amount of

target RNA in the sample.

Transcripts may also be subjected to a reverse transcriptase reaction in order to generate cDNAs which may be analyzed. For example such cDNA copies of transcripts may be analyzed for the presence of mutations. Mutational analysis includes but is not limited to, point mutations, deletions and insertions. These mutations can be detected by methods which are well-known in the art such as direct DNA sequencing (Maxam & Gilbert, 1980 *Methods Enzymol.* 65:499-559; Sanger, et al., 1977 *Proc. Natl. Acad. Sci. USA*, 74:5463-5467) and single-strand conformation polymorphism (SSCP) analysis (Leone, et al., 1993 *Oncogene*, 8:855-865). Furthermore, transcripts can be directly sequenced by using reverse transcriptase with appropriate oligodeoxyribonucleotide primers and chain terminating dideoxynucleotides (Mierendorf & Pfeffer 1987 *Methods. Enzymol.*, 15:563-566). Any reverse transcriptase can be used to perform this activity, preferably one which lacks RNase H activity, such as SuperScript II™ RNase H (Life Technologies). Lack of RNase H activity eliminates degradation of RNA molecules during the first strand cDNA synthesis, thus enabling the RNA template to be sequenced directly.

DNA probes used to detect CAR-produced RNA transcripts are synthesized or isolated in accordance with methods well known in the art as described by *Sambrook et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, COLD SPRING HARBOR LABORATORY, Cold Spring Harbor, NY (1990). The probes can be double or single-stranded DNA molecules. A double-stranded DNA probe must first be denatured in base or with heat so that it becomes single-stranded prior to hybridization to the target RNA transcripts. If base is used to denature the double-stranded probe, then it is preferred that the base is sodium hydroxide in a concentration of between 0.1 and 2.0 M, and is incubated with the probe at a temperature between 20 and 100°C for a period of between 5 and 120 minutes. More preferably, the

base is 1.25 N NaOH and is incubated with the probe for ten minutes at room temperature. If heat is used to denature the probe, then it is preferred that the probe is incubated at 90-100°C for a period between 5 and 100 minutes. More preferably, the probe is heated at a temperature of 90-100°C for 10-15 minutes.

In order to avoid renaturation of the denatured DNA probe, the RNA transcript is preferably diluted in a neutralizing buffer, or neutralizing probe diluent, and the diluted RNA is then added to the denatured DNA probe to simultaneously neutralize the base and expose the target RNA to the denatured DNA probe for hybridization. It will be understood by those skilled in the art that a neutralizing probe diluent is defined herein as a buffer that will effectively neutralize the base. Numerous neutralizing buffers are well known to those skilled in the art. Preferably, the neutralizing probe diluent is a 2-[bis(2-Hydroxyethyl) amino] ethane sulfonic acid and sodium acetate buffer (BES/sodium acetate buffer).

Base or heat treatment is not required for single-stranded DNA probes. However, because single-stranded DNA probes are usually circular molecules, having been produced from a phage such as M13 bacteriophage, base treatment of the circular DNA nicks the circles, resulting in linear single-stranded DNA probes that generally produce improved hybridization.

The DNA detection probe may be labelled with a ligand and the ligand-labelled RNA:DNA hybrid is captured onto a solid phase coated with a substrate to which the ligand will bind with specificity. The captured hybrid is then detected as described in more detail below.

It will be understood by those skilled in the art that a solid phase includes polystyrene, polyethylene, polypropylene, polycarbonate or any solid plastic material in the shape of test tubes, beads, microparticles, dip-sticks or the like. A solid phase also includes glass beads, glass test tubes and any other appropriate shape

made of glass. A functionalized solid phase such as plastic or glass that has been modified so that the surface contains carboxyl, amino, hydrazide or aldehyde groups can also be used. Therefore, any solid phase such as plastic or glass microparticles, beads, dip-sticks, test tubes or, preferably, microtiter plates can be used.

Any DNA probe used in the present invention may be labelled with at least one ligand by methods well-known to those skilled in the art including, for example, nick-translation, chemical or photochemical incorporation, and the incorporation of a ligand labelled primer into an amplified product such as a PCR product. In addition, the DNA probe may be labeled at multiple positions with one or multiple types of labels. Preferably, the DNA probe and capture probe are labelled with biotin, which binds to streptavidin; digoxigenin, which binds to anti-digoxigenin; or 2,4-dinitrophenol (DNP), which binds to anti-DNP. Fluorogens can also be used to label the probes. Examples of fluorogens include fluorescein and derivatives, phycoerythrin, allo-phycocyanin, phycocyanin, rhodamine, Texas Red or other proprietary fluorogens. The fluorogens are generally attached by chemical modification and bind to a fluorogen-specific antibody, such as anti-fluorescein. It will be understood by those skilled in the art that the probe can also be labelled by incorporation of a modified base containing any chemical group recognizable by specific antibodies. Other labels and methods of labelling nucleotide sequences for capture onto a solid phase coated with substrate are well known to those skilled in the art. A review of nucleic acid labels can be found in the article by Landegren, *et al.*, "DNA Diagnostics-Molecular Techniques and Automation", Science, 242:229-237 (1988), which is incorporated herein by reference.

Most preferably, the label is biotin, the biotin-DNA:RNA hybrids are captured on a streptavidin-coated solid phase, and the captured hybrid

is detected with an anti-DNA-RNA alkaline phosphatase conjugate. Preferably, streptavidin-coated microtiter plates are used. These plates may be coated passively or purchased commercially from Xenopore (Saddle Brook, NJ) or prepared using the methods outlined below for immobilization of anti-hybrid antibody.

The detection probe may be unlabelled and an anti-hybrid antibody, either polyclonal or monoclonal, may be immobilized on the solid phase. It will be understood by those skilled in the art that the immobilized antibody can be bound directly to the solid phase or indirectly by use of a primary binding antibody or protein, such as streptavidin or protein G, that is bound to the solid phase and which subsequently binds the anti-hybrid antibody, a derivatized anti-hybrid antibody, a functional fragment of the anti-hybrid antibody, or a derivatized functional fragment of the anti-hybrid antibody.

Excess DNA probe in the sample is preferably degraded by enzymatic treatment with RNAase-free DNAase, available from the Sigma Chemical Co., St. Louis, MO.

Any anti-hybrid antibody may be used to capture the hybrid onto the solid phase so long as the antibodies are specific for a double-stranded RNA:DNA hybrid. Such polyclonal anti-RNA:DNA hybrid antibodies may be derived from goats immunized with an RNA:DNA hybrids. Hybrid-specific antibodies maybe purified from the goat serum by affinity purification against RNA:DNA hybrid immobilized on a solid support. Monoclonal antibodies prepared using standard techniques can be used in place of the polyclonal antibodies.

The RNA:DNA hybrid antibody for capture or detection is prepared by the method of Kitawaga, Y. and Stollar, B.D., *Mol. Immunology* 19:413-420 (1982) or according to the method set forth in U.S. Patent No. 4,732,847, issued March 22, 1988 to Stuart *et al.*, both of which are incorporated herein by reference.

It will be understood by those skilled in the

art that either polyclonal or monoclonal anti-hybrid antibodies can be immobilized on the solid phase in the present assay as described below.

The anti-hybrid antibody is immobilized onto a solid phase such as a test tube surface or a 96-well microtiter plate. Immobilization of the antibody can be direct or indirect. Preferably, the solid phase is directly coated with anti-hybrid antibody in accordance with methods known to those skilled in the art or briefly described below. The antibody can also be biotinylated and subsequently immobilized on streptavidin coated surfaces, or modified by other means to covalently bind to the solid phase. Solubilized biotinylated antibody can be immobilized on the streptavidin coated surfaces before capture of the hybridized samples as described below or in conjunction with the addition of the hybridized samples as described below or in conjunction with the addition of the hybridized samples to simultaneously immobilize the biotinylated antibody and capture the hybrids.

More preferably, the antibody is attached to the solid phase in accordance with the method of Fleminger, G., et al., *Appl. Biochem. Biotech.* 23:123-137 (1990), by oxidizing the carbohydrate portion of the antibody with periodate to yield reactive aldehyde groups. The aldehyde groups are then reacted with a hydrazide-modified solid phase such as MicroBind-HZ™ microtiter plates available from Dynatech Laboratories (Chantilly, VA). Passive coating of the antibody according to the well known method of Esser, P., Nunc Bulletin No. 6 (Nov. 1988) (Nunc, Roskilde, Denmark) is also acceptable.

Alternatively, Ventrex Star™ tubes (Ventrex Laboratories Inc., Portland, ME) are coated with streptavidin by the method of Haun, M. and Wasi, S., *Anal. Biochem.* 191:337-342 (1990). After binding of streptavidin, the biotinylated goat polyclonal antibody described above, or otherwise produced by methods known to those skilled in the art, is bound to the immobilized

streptavidin. Following antibody binding, the solid matrix can be post-coated with a detergent such as Tween™-20 and sucrose to block unbound sites on the surface and stabilize the bound proteins as described by Esser, P., Nunc Bulletin No. 8, pp. 1-5 (Dec. 1990) and Nunc Bulletin No. 9, pp. 1-4 (June 1991) (Nunc, Roskilde, Denmark) and Ansari, et al. *J. Immunol. Methods* 84:117-124 (1985). Preferably, each surface is coated with between 10 ng and 100 μg biotinylated antibody. Most preferably each surface is coated with approximately 250 ng of biotinylated antibody.

As discussed below, the solid phase can be coated with functional antibody fragments or derivatized functional fragments of the anti-hybrid antibody.

The CAR-produced RNA:DNA probe hybrids are exposed to the solid phase, which has been coated with either a substrate that binds with specificity to the ligand or ligand-conjugated probe or an anti-hybrid antibody, as described above, for a sufficient amount of time to allow binding or capture of the hybrid by the immobilized antibodies or substrate. The hybrids are bound to the immobilized antibodies or substrate by incubation for about five minutes to about twenty-four hours at about 15°C to 65°C on a platform shaker with a shaking speed of 0 to 1500 rpm. Preferably, the incubation time is about 30 to about 120 minutes at about 20°C to 40°C, with shaking at 300 to 1200 rpm. More preferably, capture occurs with incubation at one hour at room temperature with vigorous shaking on a rotary platform shaker with a rotary shaking speed between approximately 300 and 1000 rpm. It will be understood by those skilled in the art that the incubation time, temperature, and shaking can be varied to achieve alternative capture kinetics as desired.

Hybridization is detected by conventional means well known in the art such as with a direct labelled polyclonal or monoclonal antibody specific for the hybrid

or a labelled antibody. Alternatively, if the probe is labelled with a ligand as described above in the preferred embodiment, the hybrid can be detected with either a labelled anti-hybrid antibody or a labelled substrate, such as a streptavidin-alkaline phosphate conjugate. In the preferred embodiment, the target RNA is hybridized to a labelled probe, the hybrid is captured onto a substrate-coated solid phase, and the captured hybrid is detected onto a substrate-coated solid phase, and the captured hybrid is detected with a labelled anti-hybrid antibody.

Most preferably, the label of the anti-hybrid antibody is an enzyme, a fluorescent molecule or a biotin-avidin conjugate and is non-radioactive. The label can then be detected by conventional means well known in the art such as a colorimeter, a luminometer, or a fluorescence detector. The preferred label is alkaline phosphatase.

Detection of captured hybrid is preferably achieved by binding the above-described conjugated anti-hybrid molecule to the hybrid during incubation. Surfaces are then washed with the above-described wash buffer to remove any excess conjugate. Preferably, five manual washes are performed using either an Eppendorf™ Repeat Pipettor with a 50 ml Combitip™ (Eppendorf, Hamburg, Germany), a Corning repeat syringe (Corning, Corning, NY), a simple pump regulated by a variostat, or by gravity flow from a reservoir with attached tubing. Commercially available tube washing systems available from Source Scientific Systems (Garden Grove, CA) can also be used.

As described above, captured hybrid can also be detected with a direct labelled DNA probe, such as an enzyme-conjugated hybridization probe, or a hapten-modified probe that is subsequently detected by a labelled anti-hapten antibody.

Bound conjugate is subsequently detected by colorimetry or chemiluminescence as described at Coutlee, *et al., J. Clin. Microbiol.* 27:1002-1007 (1989).

Preferably, bound alkaline phosphatase conjugate is detected by chemiluminescence with a reagent such as a Lumi-Phos™ S30 reagent (Lumigen, Detroit, MI) using a detector such as an E/Lumina™ luminometer (Source Scientific Systems, Inc., Garden Grove, CA) or an Optocomp I™ Luminometer (MGM Instruments, Harden, CT).

A further embodiment of the present invention relates to the CAR procedure carried out as an automated process. Such a procedure may use an automated device for carrying out hybridization, polymerase extension, transcription and detection reactions in one or more vessels. This process is capable of analyzing multiple samples sequentially or simultaneously. The process may be automated in such a way as to include the use of robotics, such as the Biomek 2000™ (Beckman Instruments, Fullerton, CA), Plato 3300™ or Plato 1300™ (Rosys, Wilmington, DE) or LABTECH™ (Biochem Immunosystems, Allentown, PA). An automated thermoregulator combined with robotics may be particularly advantageous in an automated system for CAR, which could use a system such as a Robocycler 96™ or Robocycler 40™ (Stratagene, LaJolla, CA). Other systems for automating the CAR method are known in the art and are within the scope of the CAR invention.

One non-radioactive CAR assay kit contains the necessary devices and reagents for performing a CAR amplification reaction and a non-radioactive hybridization assay, as described above including an appropriate sample collection device, such as a dacron swab for exfoliated cell sample collection; sample transport medium for stabilization of the sample during transport to the laboratory for analysis; a promoter-primer for a specific nucleic acid target; a trimming probe or at least one 3'-5' exonuclease or at least one polymerase having 3' exonuclease activity for trimming back any sequence 3' to the target region in a nucleic acid; one or more DNA polymerases; RNA polymerase(s); one or more probes

specific for the transcript to be detected; neutralizing probe diluent; anti-hybrid antibody- or substrate-coated test tubes or microtiter wells; a nuclease such as RNase, preferably contained in a solution also containing a conjugated anti-hybrid antibody that can be detected by conventional means; and any necessary controls.

The kit should contain a negative control and a positive control for each detection probe. Preferably, the negative control is enzymatically prepared RNA of a sequence that is not complementary to the detection probe. The positive control preferably contains enzymatically prepared RNA that is complementary to the probe.

In general, the assay can be used to detect as little as 1 pg RNA per ml of specimen with a typical specimen volume of 100 μl.

The following examples illustrate use of the present amplification method and detection assay and kit. These examples are offered by way of illustration, and are not intended to limit the scope of the invention in any manner. All references described herein are expressly incorporated *in toto* by reference.

EXAMPLE 1

Synthetic Promoter

A 65 base oligonucleotide promoter-primer, containing the T7 RNA polymerase promoter core sequence and flanked by 20 base regions complementary to HIV-1, was chemically synthesized.

HIV-20 mer T7 Promoter HIV-20 mer

+1

5'-**AGTAAAGCCAGAGGAGATCT**TAATACGACTCACTATAGG**GAATTCCTGCAGAATGGGATAGATTG**-3'

The HIV -1 regions of the promoter-primer are non-contiguous with respect to HIV and extend from base 665 to base 684 and base 1415 in the *gag* region of the genome (Adachi A, et al. 1986, *J. Virol* 59:284-291 [Accession # M19921]). The consensus sequence of the T7

RNA polymerase promoter region is well characterized (Oakley, J.L. and Coleman, J.E. 1977), *Proc. Natl. Acad. Sci.* USA **74**:4266-4270; Dunn, J.J. and Studier, F.W. 1983 *J Mol Biol* **166**:477-535) and is functional only when double-stranded (Milligan, J.F., et al. 1987. *Nuc Acids Res* **15**:8783-8799). The single-stranded promoter-primer was therefore made into a duplex, by combined 3'→5' exonuclease/5'→3' DNA polymerase enzymatic activities, prior to RNA synthesis. The sequence of the promoter-primer oligonucleotide includes the T7 promoter conserved core region extending 17 bases upstream of the transcriptional initiation site (designated + 1). The GGGA nucleotide sequence, immediately downstream of the 17 base core region, is the preferred site for transcription initiation (Milligan, J.F., et al. 1987, *Nuc Acids Res* **15**:8783-8799). The nucleotides between the promoter region and the HIV-1 sequences generate an *Eco* RI restriction site that was inserted for convenience.

In order to illustrate the CAR method, a plasmid was constructed and modified to generate various DNA model targets. The DNA used to generate the different targets is a 1181 bp *Hind III* fragment from the *gag* region of HIV-1. This fragment was subcloned from plasmid pNL4-3 (Adachi, et al. 1986, *J Virol* **59**:284-291) into the *Hind III* site of pIC20H (Marsh, J. et al. 1984. *Gene* **32**:481-485) to create plasmid pRK15 (Fig. 2). Digestion of plasmid pRK15 with different restriction endonucleases, followed by gel purification of the fragments, allows a variety of different target types to be formed. These are diagrammed in Figure 3 and represent the structures generated after hybridization of the promoter-primer with each of the target fragments. Both linear and circular structures can be formed depending on the particular restriction endonuclease used to cut pRK15. For both linear and circular hybrid structures, any single stranded sequence 3' to the target region (also referred to herein as "3' end tails") may be removed by 3'→5' exonuclease

prior to synthesizing a double stranded promoter region. Depending on the target DNA, the length of the 3' end tails were from 0 to 736 bases. The enzymes used to digest pRK15 to generate each of the targets is shown in Table 1.

Table 1. Profile of pRK15 Generated Targets for CAR.

Target	Type	Enzyme(s)	5' Tail length	3' Tail Length
1	circular	*Bgl II + Pst I*	0	0
2	circular	*Pst I*	736	0
3	circular	*Bgl II*	0	736
4	circular	*Nsi I*	568	168
5	circular	*Bss HII*	32	704
6	linear	*Pst I + Sca I*	NA	0
7	linear	*Nsi I + Sca I*	NA	168
8	linear	*Bss HII + Sca I*	NA	704

Hybridization and DNA Polymerase Reactions

Each of the targets (0, 5 x 10^4, 5 x 10^6, 5 x 10^7 or 5 x 10^8 molecules per reaction) were added to a hybridization mix comprising of 1 X Vent™ polymerase buffer (New England Biolabs)(10 mM KCl, 10 mM $(NH_4)SO_4$, 20 mM Tris-HCl [pH 8.8], 2 mM $MgSO_4$ and 0.1% Triton-X-100), 55 nM of promoter-primer, 0.5 mM of each dNTP and 2 units of Vent™ (exo-) DNA polymerase (New England Biolabs), in a 15 μl final volume. The DNA was first denatured at 100°C for 5 minutes, and later hybridized with the promoter-primer at 48°C for 30 minutes. At the end of this period, 1 unit (1 μl) of Vent™ (exo+) DNA polymerase (NEB) was added to each tube. The tubes were then incubated for 30 minutes at 75°C and the reactions terminated by diluting the samples to 100 μl with H_2O. The samples were then extracted with phenol/chloroform/iso-amyl alcohol

(49.5/49.5/1) and further diluted with H_2O to 500 µl volumes. All DNA samples were concentrated to 10 µl final volumes with Microcon-30 filtration units (Amicon).

Transcription Reactions

The DNA samples (10 µl) were transcribed in 50 µl final volumes, each containing 10 mM DTT, 2 mM of each NTP, 40 mM Tris, pH 8.0, 8 mM $MgCl_2$, 75 mM NaCl, 100 µg/ml BSA, 5 units/µl RNAsin (Promega), 0.025 units/µl of inorganic pyrophosphatase (Sigma) and 6 units/µl of T7 RNA polymerase (Pharmacia). The transcription reactions were performed at 37°C for two hours.

EXAMPLE 2

Detection of Amplified RNA Transcripts

A 5' -end biotinylated DNA probe was synthesized by polymerase chain reaction using a single biotinylated primer (positions 864-885 of Promega's pGEM3Z) and a non-biotinylated primer (position 180-202 of Promega's pGEM3Z). The DNA sequence of pRK15 and pGEM3Z are identical within these regions, but the former was used as the PCR template. Amplification was performed with the reagents from a Perkin-Elmer Cetus Gene Amp™ kit, 2 mM $MgCl_2$, and 0.5 µM of each primer, using a Perkin Elmer DNA Thermal Cycler. The thermal cycling profile used for PCR amplification involved an initial 3 minute denaturation step at 95°C, followed by 40 amplification cycles (1 minute at 94°C, 2 minutes at 55°C and 2 minutes at 72°C), and ended with a 10 minute extension at 72°C. The 704 base PCR generated product was purified using the Magic™ PCR Prep DNA Purification System (Promega).

Detection of CAR synthesized RNA was measured using a modification of the SHARP Signal™ System (Digene Diagnostics, Inc., Silver Spring, MD). A 5 µl aliquot of biotinylated DNA was denatured in a mix containing 5 µl of SHARP™ sample diluent and 25 µl of SHARP™ denaturing reagent (Digene Diagnostics, Inc., MD) at room temperature for 10 minutes. After initial incubation period, 25 µl of SHARP™ probe diluent (Digene Diagnostics, Inc., MD) was

added to the reaction and the entire volume (60 µl) transferred into the transcription reaction sample tube. It is noted that the biotinylated DNA probe was present in the hybridization cocktail at a 1 nM final concentration. Hybridization was performed at 65°C for 30 minutes. Following hybridization, the mixture was transferred to streptavidin coated plates and shaken for 30 minutes at 1100 rpm at room temperature. The mixture was decanted from the wells, 100 µl of SHARP™ detection reagent (Digene Diagnostics, Inc., MD) added and the plates were allowed to shake at 1100 rpm for 30 minutes at room temperature. The wells were then washed 5 times with SHARP™ wash solution (Digene Diagnostics, Inc., MD), twice with H_2O and blotted on paper towels to remove excess liquid. Finally, 100 µl of SHARP™ substrate was added and the plates incubated at 37°C for 1-12 hours before reading the absorbance at 410 nM in a plate reader (Bio-Rad).

EXAMPLE 3

Effect of 3' Tail and Template Type on CAR

The effects of the 3' tail and template type on CAR are summarized in Table 2. The background was represented by sample 1 which is the zero target control. Comparing the signal obtained from circular target (samples 2-6) with the obtained from linear target 6 (samples 7-11), or circular target 4 (samples 12-16) with linear target 7 (samples 7-21), indicates that there was no significant difference between linear and circular target amplification. However, when the signals from targets that lack 3' tails were compared with those obtained from the targets which required exonucleolytic removal of the 3' tail, a large difference in amplification was observed. The signal was approximately 100 fold less for those targets that have a 3' tail. These data indicate that exonucleolytic removal of the 3' tail, prior to synthesis of a double stranded promoter by DNA polymerase was limiting the reaction.

Table 2. Effect of 3' Tail and Template Type on CAR

Sample	A_{410}
1. Probe without target	0.003
Target #1 (circular, no 3' tail)	
2. 5×10^8	****
3. 5×10^7	****
4. 5×10^6	****
5. 5×10^5	0.254
6. 5×10^4	0.053
Target #6 (linear, no 3' tail)	
7. 5×10^8	****
8. 5×10^7	****
9. 5×10^6	****
10. 5×10^5	0.493
11. 5×10^4	0.013
Target #4 (Circular, 168 base 3' tail)	
12. 5×10^8	****
13. 5×10^7	0.403
14. 5×10^6	0.029
15. 5×10^5	0.010
16. 5×10^4	0.009
Target #7 (linear, 168 base 3' tail)	
17. 5×10^8	****
18. 5×10^7	0.281
19. 5×10^6	0.042
20. 5×10^5	0.011
21. 5×10^4	0.005
**** = signal > 2.5000	

EXAMPLE 4

Effect of Alternative DNA Polymerases

There are several alternative combinations of DNA polymerases and exonucleases that can be used, either simultaneously or sequentially, to generate a functional double stranded promoter in the CAR method. Both thermostable and non-thermostable enzymes can be used depending on the reaction conditions. The primary requirement in this embodiment of the invention is that the promoter region remains non-functional, unless the specific target is present. As an alternative to the previously described methods, T7 DNA polymerase was used in place of Vent™ (exo+) DNA polymerase in the second step of the DNA synthesis reaction. The reaction conditions as described in Example 1 remained virtually unchanged, except for the changes which follow: Target 7 (see Fig. 3) was used at amounts from 5×10^6 to 5×10^8. This DNA/promoter-primer hybrid structure leaves a 3' tail (168 nucleotides long). In the first step of the hybridization reaction described in Example 1, Vent™ (exo-) DNA polymerase was replaced with Deep Vent™ (exo-) DNA polymerase (New England Biolabs). In the second step of the reaction, either no additional DNA polymerase was added and the reaction incubated at 75°C, or Deep Vent™ (exo+) DNA polymerase was added at 75°C, or T7 DNA polymerase was added and the reaction was incubated at 37°C.

The results are depicted in Table 3. The data indicates that using T7 DNA polymerase increases the signal 5 to 10 fold over that obtained with Deep Vent™ (exo+) DNA Polymerase. It can be hypothesized that T7 DNA polymerase has a higher, or more processive, 3'→5' exonuclease activity than Deep Vent™ (exo+) DNA polymerase, and was therefore able to remove the 3' tail more efficiently. Removal of the 3' tail enabled the 5'→3' polymerase activity of the enzyme to fill the complimentary strand, thus generating the double stranded

T7 promoter region, which is ultimately required for successful transcription by T7 RNA polymerase.

Table 3. Effect of DNA Polymerase on CAR Activity.

	Sample	2nd DNA Polymerase	A_{410}
1.	5×10^8	none	1.584
2.	5×10^7	none	0.199
3.	5×10^6	none	0.031
4.	5×10^8	Deep Vent (exo+)	****
5.	5×10^7	Deep Vent (exo+)	0.519
6.	5×10^6	Deep Vent (exo+)	0.047
7.	5×10^8	T7 DNA Polymerase	****
8.	5×10^7	T7 DNA Polymerase	****
9.	5×10^6	T7 DNA Polymerase	0.490
10.	0	none	0.005
**** = signal > 2.5000			

Hybridization and DNA polymerase reaction conditions have been optimized in a two step reaction. First, hybridization was performed in the presence of the Deep Vent™ (exo-) DNA polymerase (first DNA polymerase). The lack of 3' exonuclease activity in this first step prevents degradation of the single stranded promoter, while at the same time, the hybridization process is enhanced by the extension of the 3' end of the promoter-probe during the annealing process (longer probe further stabilizes the hybrid). In the second step, or Deep Vent™ (exo+), or T7 DNA polymerase is added. The 2nd DNA polymerase has 3'→5' exonuclease (removing the 3'-end tail) and 5'→ 3' polymerizing activity, resulting in the synthesis of double stranded DNA transcription targets.

CAR technology, using the previously mentioned T7 DNA polymerase method was repeated using less input target DNA. The results of this experiment are summarized in Table 4. These data reveal the ability of the system to easily detect 5×10^5 input DNA target molecules.

Table 4. Effect of Low Target Levels on CAR

Sample		A_{410}
1.	Probe without target	-0.12
2.	5×10^7	****
3.	5×10^6	1.481

4.	5×10^5	0.187
5.	5×10^4	0.035
6.	5×10^3	0.010
**** = signal > 2.500		

EXAMPLE 5

Hepatitis B Virus CAR Model System

The Human Hepatitis B Virus (HBV) genome is small, approx. 3200bp, and composed of partially double-stranded DNA. The substrate for viral transcription *in vivo* is the complete (-) DNA strand. The (-) strand DNA is convenient to hybridize with a CAR promoter-primer due to the lack of a full (+) strand DNA. The fact that the entire DNA sequence of HBV is transcribed as a single message *in vivo* (which is detected via DNA:RNA hybrid formation), coupled with the above mentioned genomic features, makes HBV a suitable model target for CAR technology.

Synthetic Promoter

Two 75 base oligonucleotide promoter-primers, containing the T7 RNA polymerase promoter core sequence and flanked by 25 base regions complementary of HBV sequence were chemically synthesized.

Promoter-Primer HBV-32 (For circular CAR using the 1.6 kb HBV

5'-P-CTCCCCGTCTGTGCCTTCTCATCTG**TAATACGACTCACTATAGGGAATTC**CAG
AGTCTAGACTCGTGGTGGAC-S-T-S-T3'

Promoter-Primer HBV-31 (For circular and linear CAR using the entire genome of HBV)

5'-P-CTCCCCGTCTGTGCCTTCTCATCTG**TAATACGACTCACTATAGGGAATTC**ATC
GCCGCGTCGCAGAAGATCTC-S-A-S-A-3'

Alignment of HBV DNA sequences from the major subtypes (adw2, adw, adr1, adr2, ayr, ayw1 and ayw2) indicated stretches of highly conserved nucleotide sequences which were used to generate the above CAR promoter-primers. The HBV regions of the promoter-primer are non-contiguous with respect to HBV and are conserved throughout all the major HBV DNA subtypes analyzed. This permitted amplification and detection of different HBV subtypes using the CAR method. The HBV sequences within the promoter-primer extended from base 1547 to base 1571 (X gene coding region) and base 224 to 268 (HBsAg coding region) of the genome for the HBV-32 promoter-32 promoter-probe, and from base 1547 to base 1571 (X gene coding region) and base 2415 to 2439 (HBcAg coding region) for the HBV-31 promoter probe (Anneke K. Raney and Alan McLachlan, The Biology Hepatitis B Virus, in *Molecular Biology of the Hepatitis B Virus*, Alan McLachlan, Eds., CRC Press Inc., Boca Raton, Florida, 1991, 5-13). The consensus sequence of the T7 RNA polymerase promoter region is well characterized (Oakley, J.L. and Coleman, J.E. 1977. *Proc. Nat'l. Acad. Sci. USA* 74:4266-4270; Dunn, J.J. and Studier, F.W. 1983. *J Mol Biol* 166:477-535) and is functional only when double-stranded (Milligan, J.F., et al. 1987. *Nuc Acids Res* 15:8783-8799). The single-stranded promoter-primer is preferably made into a duplex, by combined 3'↦5' exonuclease/5'↦3' DNA polymerase enzymatic activities, prior to DNA synthesis. The sequence of the promoter-primer oligonucleotide included the T7 promoter conserved core region extending 17 bases upstream of the transcriptional initiation site (designated + 1). The GGGA nucleotide sequence, immediately downstream of the 17 base core region, is the preferred site for transcription initiation (Milligan, J.F., et al. 1987 *Nuc Acids Res* **15**:8783-8799). The nucleotides between the promoter region and the HBV sequences generated an *EcoRI* restriction site that was inserted for convenience.

The 75-mer promoter-primers also had a 5'-end phosphate and 3'-end phosphorothioate linkages between the last, second to last and third to last nucleotides ("blocked ends"). The two tandem phosphorothioate linkages prevented 3'-5' exonucleolytic processive cleavage of the promoter-primers by the DNA polymerase without interfering with the 5'-3' polymerizing activity of the enzyme.

Using two different promoter-primers hybridized circles of different dimensions were generated, with 5' and/or 3' tails varying in length, depending on the size and sequence of the target DNA to be copied and transcribed via CAR.

EXAMPLE 6

In order to illustrate circular CAR, two plasmids were constructed and modified to generate various DNA targets. The DNA used to generate the different targets was a 1581 bp *EcoRI/BsiHKAI* fragment from HBV *ayw* and *adw2* strains. After double digestion of pGEM3Z with *EcoRI* and *PstI*, these fragments were cloned into plasmid pGEM3Z (Promega) to create plasmids pADRAYW and pADRADW2 respectively. Digestion of plasmids pADRAYW and/or pADRADW2 with different restriction endonucleases, followed by gel purification of the fragments, allowed a variety of different target types to be formed. Both linear and circular structures can be formed depending on the particular restriction endonuclease used to cut pADRAYW and/or pADRADW2. For both linear and circular hybrid structures the 3' end tail of the target, if present, must be removed by a 3'→5' exonuclease prior to synthesizing a double stranded promoter region.

Hybridization, DNA Polymerase and RNA Polymerase Reactions (single buffer/single tube format)

The reaction buffer contained salts, DTT, four dNTPs (dATP, dGTP, dCTP, dTTP) four NTPs (ATP, GTP, CTP, UTP), one or more suitable RNase inhibitors and one or

more suitable carrier proteins. The final reaction volume was 25 μl and contained the reaction buffer, target DNA (10^9 molecules) and promoter-primer (10^{13} molecules).

The reaction buffer, DNA and promoter-primer were mixed together and heated for 1 min. at 100°C. The heated mixture was then allowed to cool to 37°C for 10 min. Five units of *E.coli* DNA polymerase I and 5 units of T7 RNA polymerase were added to the mixture, and incubation at 37°C for two hours followed.

Detection of CAR synthesized RNA was performed by running the reactions on a denaturing formaldehyde gel and staining with ethidium bromide to visualize the products. Specific RNA transcripts were observed, thus demonstrating the applicability of the CAR method to the HBV model system.

Modifications and variations of the continuous amplification reaction and corresponding kits will be obvious to those skilled in the art from the foregoing detailed description of the invention. Such modifications and variations are intended to come within the scope of the appended claims.

We claim:

1. A method of amplifying a target nucleic acid comprising the steps of:

providing a single-stranded nucleic acid containing a target region;

hybridizing the target region of said nucleic acid to a promoter-primer having a central promoter portion and two regions homologous to non-contiguous portions of the target region forming a circular hybrid;

trimming back single-stranded sequence 3' to the target region generating a flushed 3' end of said hybrid;

extending 3' ends of the target region and the promoter-primer forming a double-stranded intermediate; and

transcribing the double-stranded intermediate producing many RNA transcripts from each target region.

2. A method according to claim 1 further comprising:

denaturing a double stranded nucleic acid, thereby providing a single-stranded nucleic acid.

3. A method according to claim 2 wherein denaturing comprises:

treating said nucleic acid with a base; and

neutralizing with a hybridization neutralization buffer.

4. A method according to claim 1 wherein the single stranded nucleic acid is hybridized to a capture probe.

5. A method according to claim 1 wherein the trimming and extending steps are carried out using at least one DNA polymerase having exonuclease activity.

6. A method according to claim 1 wherein the trimming and extending steps are carried out using at least one DNA polymerase and at least one exonuclease.

7. A method according to claim 1 wherein the promoter-primer comprises modified nucleotides or phosphodiester linkages in at least an ultimate 3' end position.

8. A method of detecting a nucleic acid containing a target region comprising the steps of:

providing a single-stranded nucleic acid comprising said target region;

hybridizing said nucleic acid to a single stranded promoter-primer having a 5' promoter portion and a 3' primer portion, said 3' primer portion comprising a sequence complementary to a 3' portion of said target region, said 3' primer portion further comprising at least one modified nucleotide or phosphodiester linkage, to form a hybrid;

trimming back single-stranded sequence 3' to the target region generating a flushed 3' end of said hybrid;

extending 3' ends of said target region and the promoter-primer forming a double-stranded intermediate;

transcribing the double-stranded intermediate producing many RNA transcripts from each target region; and

detecting RNA transcripts.

9. A method according to claim 8 further comprising:

denaturing a double stranded nucleic acid, thereby providing the single-stranded nucleic acid.

10. A method according to claim 9 wherein denaturing comprises:

treating said nucleic acid with a base; and

neutralizing with a hybridization neutralization buffer.

11. A method according to claim 8 wherein the single stranded nucleic acid is hybridized to a capture probe.

12. A method according to claim 8 wherein the trimming and extending steps are carried out using at least one DNA polymerase having exonuclease activity.

13. A method according to claim 8 wherein the trimming and extending steps are carried out using at least one DNA polymerase and at least one exonuclease.

14. A method of amplifying a nucleic acid containing a target region comprising the steps of:

providing a single-stranded nucleic acid containing said target region;

hybridizing said nucleic acid to a trimming probe comprising a sequence complementary to a 3' junction of said target region forming a functional restriction endonuclease recognition site hybrid;

digesting restriction endonuclease recognition site hybrid with a corresponding restriction endonuclease thereby forming a trimmed target nucleic acid;

denaturing trimmed target nucleic acid to remove remaining portion of said trimming probe forming a trimmed single-stranded nucleic acid;

hybridizing said trimmed single-stranded nucleic acid to a partially double-stranded promoter-primer having a double-stranded promoter portion and a single-stranded primer sequence, said sequence being complementary to trimmed 3' end region of said target nucleic acid; wherein the double-stranded promoter portion directly abutts trimmed 3' end of said target nucleic acid upon hybridization forming a partially double stranded hybrid; and

transcribing said partially double-stranded hybrid producing many RNA transcripts from each target nucleic acid.

15. A method according to claim 14 further comprising:

ligating the primer portion of said partially double stranded hybrid to 3' end of said target

nucleic acid.

16. A method according to claim 14 or 15 further comprising:

extending the partially double stranded hybrid prior to said transcription step.

17. A method according to claim 14 further comprising:

denaturing a double stranded nucleic acid, thereby providing a single-stranded nucleic acid.

18. A method according to claim 17 wherein denaturing comprises:

treating said nucleic acid with a base; and

neutralizing with a hybridization neutralization buffer.

19. A method according to claim 14 wherein the single stranded nucleic acid is hybridized to a capture probe.

20. A method according to claim 14 wherein said trimming probe carries at least one ligand and wherein said method further comprises the steps of:

binding said trimming probe hybridized to said nucleic acid to a solid matrix carrying a capturing agent for said ligand thereby immobilizing said restriction endonuclease recognition site hybrid;

washing said matrix;

digesting said restriction endonuclease recognition site hybrid with a corresponding restriction endonuclease thereby forming a non-immobilized trimmed target nucleic acid; and

separating non-immobilized target nucleic acid from solid matrix.

21. The method of claim 8 wherein the RNA transcripts are detected by a method comprising the steps of:

a. hybridizing the RNA transcript to a complementary DNA probe to form a double-stranded hybrid;

b. capturing the hybrid of step (a) onto

a solid phase to form a bound hybrid;

c. eliminating non-hybridized nucleic acid; and

d. detecting the bound hybrid.

22. The method of claim 9 wherein the DNA probe is labelled with at least one ligand.

23. The method of claim 21 wherein the DNA probe is biotinylated and the solid phase is coated with streptavidin.

24. The method of claim 21 wherein an anti-hybrid antibody or anti-hybrid fragment has been immobilized to the solid phase, wherein the antibody or antibody fragment specifically binds to a component of the double-stranded hybrid.

25. The method of claim 21 wherein the probe is double-stranded DNA, comprising the additional step of treating the probe with a base to form single-stranded DNA prior to the hybridization step.

26. A kit for amplification of nucleic acid in a biological sample comprising:

- a promoter-primer having a central promoter portion and two regions homologous to non- contiguous sequences in the target region;
- a trimming back agent;
- a nucleic acid polymerase;
- an RNA polymerase; and
- a sample transport medium for stabilization of the biological sample.

27. A kit for amplification of nucleic acid in a biological sample comprising:

- a promoter-primer having a 5′ promoter portion and a 3′ primer portion, said primer portion comprising a sequence complementary to a 3′ portion of said target region, said promoter-primer

further comprising at least one modified nucleotide or phosphodiester linkage;

- a trimming back agent;
- a nucleic acid polymerase;
- an RNA polymerase; and
- a sample transport medium for stabilization of the biological sample.

28. A kit for amplification and detection of nucleic acid in a biological sample comprising the components of claims 26 or 27, further comprising the components of:

- a DNA detection probe complementary to the target RNA sequence for formation of a double-stranded nucleic acid hybrid;
- a neutralizing probe diluent for diluting and neutralizing the treated probe;
- a solid phase coated with a coating to which a hybrid formed by hybridization of the probe and the target nucleic acid sequence, will bind; and
- means for detecting the hybrid formed by hybridization of the probe and the target nucleic acid sequence.

29. The kit of claim 28 wherein the solid phase is coated with streptavidin and the probe is biotinylated.

30. The kit of claim 28 wherein the solid phase is coated with an anti-hybrid antibody or an anti-hybrid antibody fragment, wherein the antibody or antibody fragment specifically binds to a component of the double-stranded hybrid.

31. The kit of claim 28 wherein the means for eliminating the non-hybridized probe is DNase.

32. The kit of claim 28 wherein the detecting

means is an anti-hybrid antibody or an anti-hybrid antibody fragment, wherein the antibody or antibody fragment specifically binds to a component of the double-stranded hybrid and is detectable.

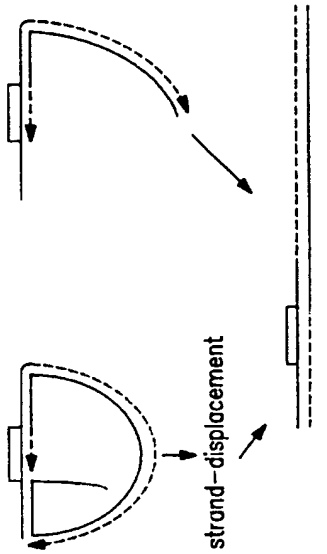


FIG. IC

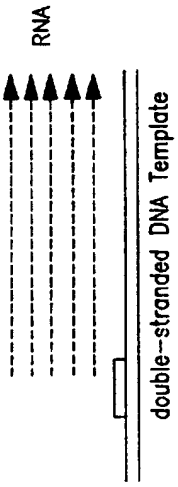


FIG. ID

FIG. IE

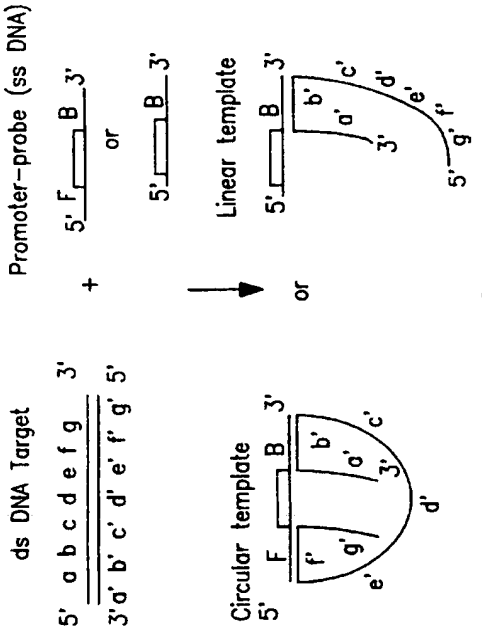


FIG. IA

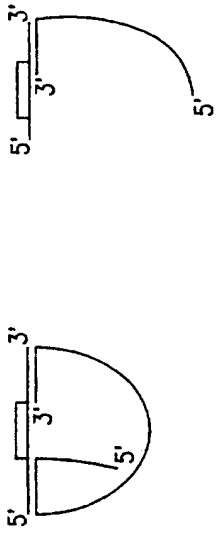
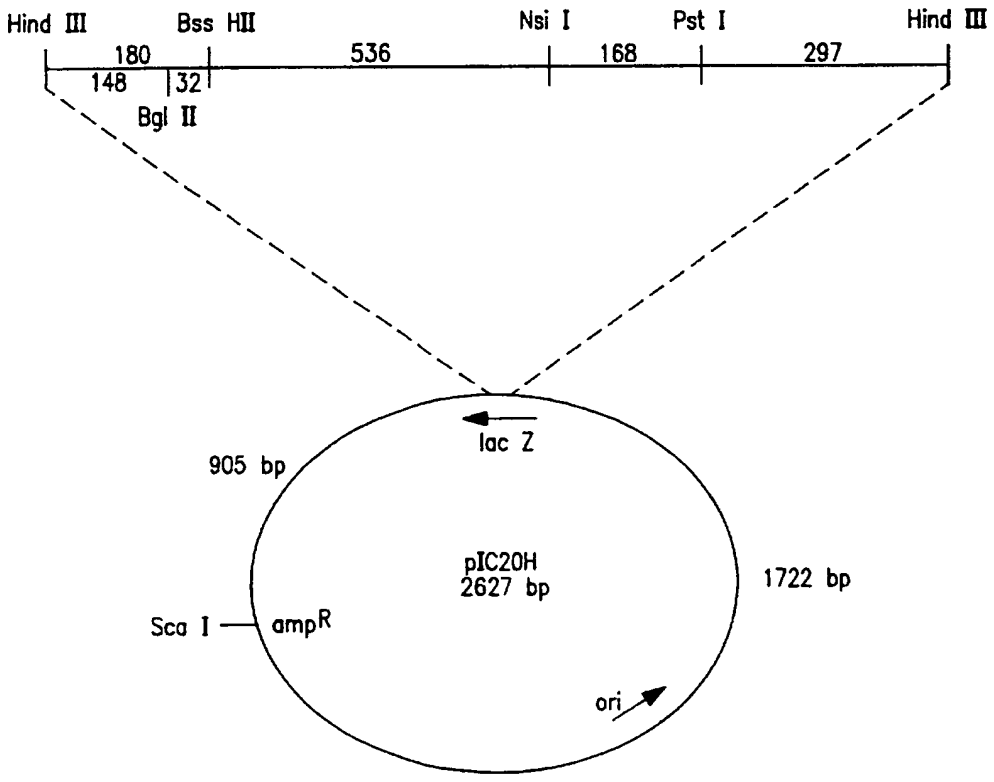


FIG. IB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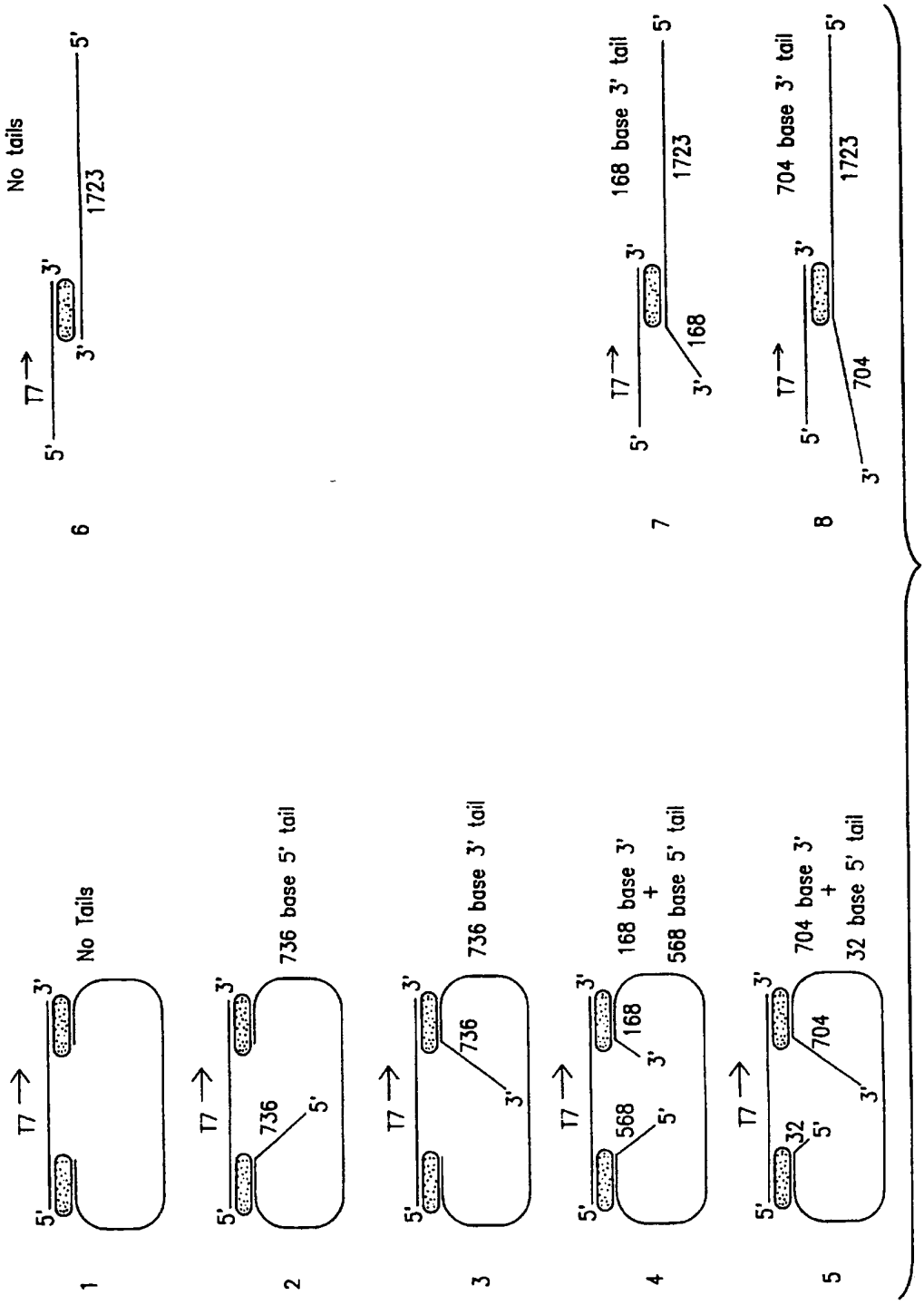
Sca I
ampR
ori

FIG. 2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T7
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3'
1723
6
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168
7
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8
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736
2
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+
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4
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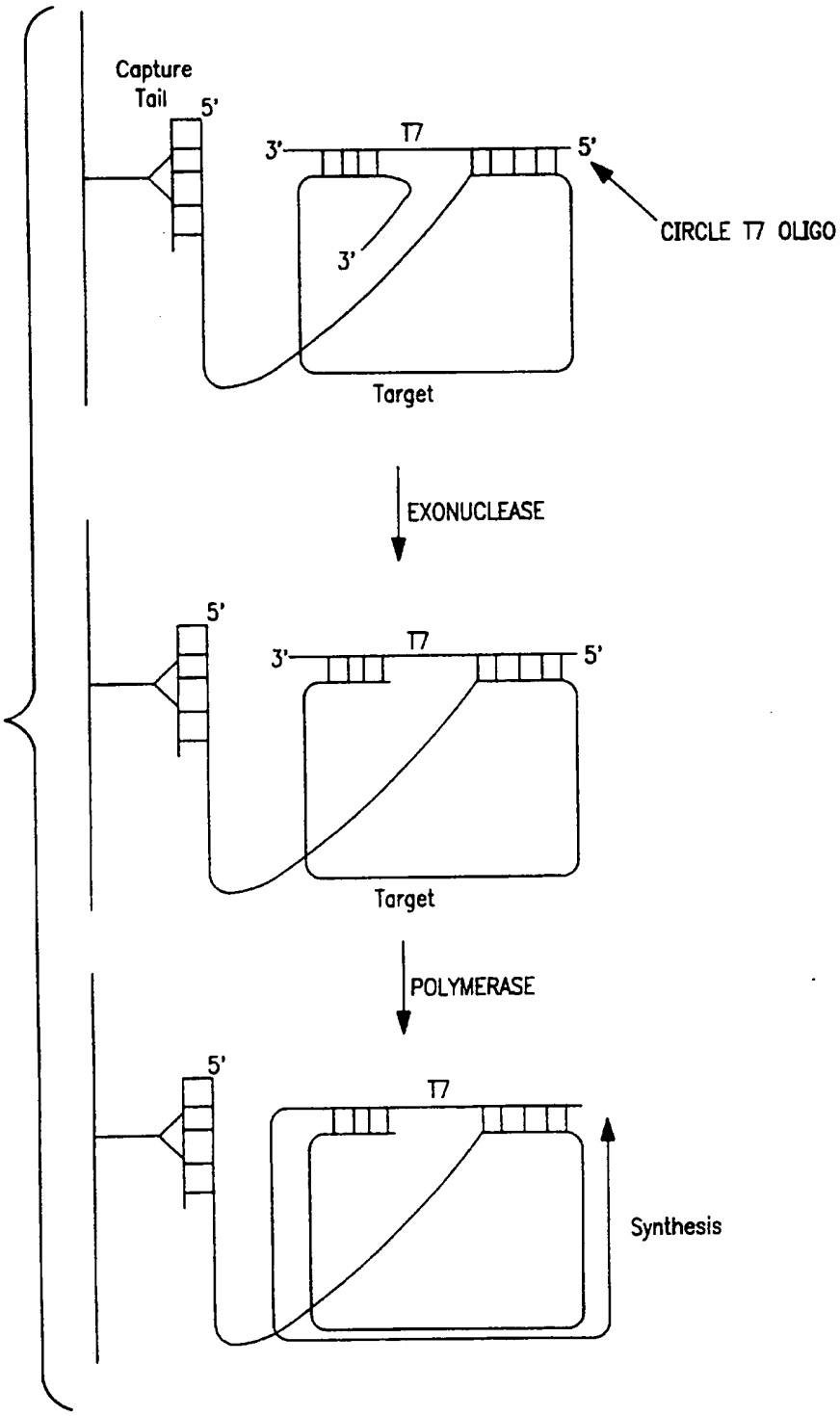
32
5

FIG. 3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POLYMERASE
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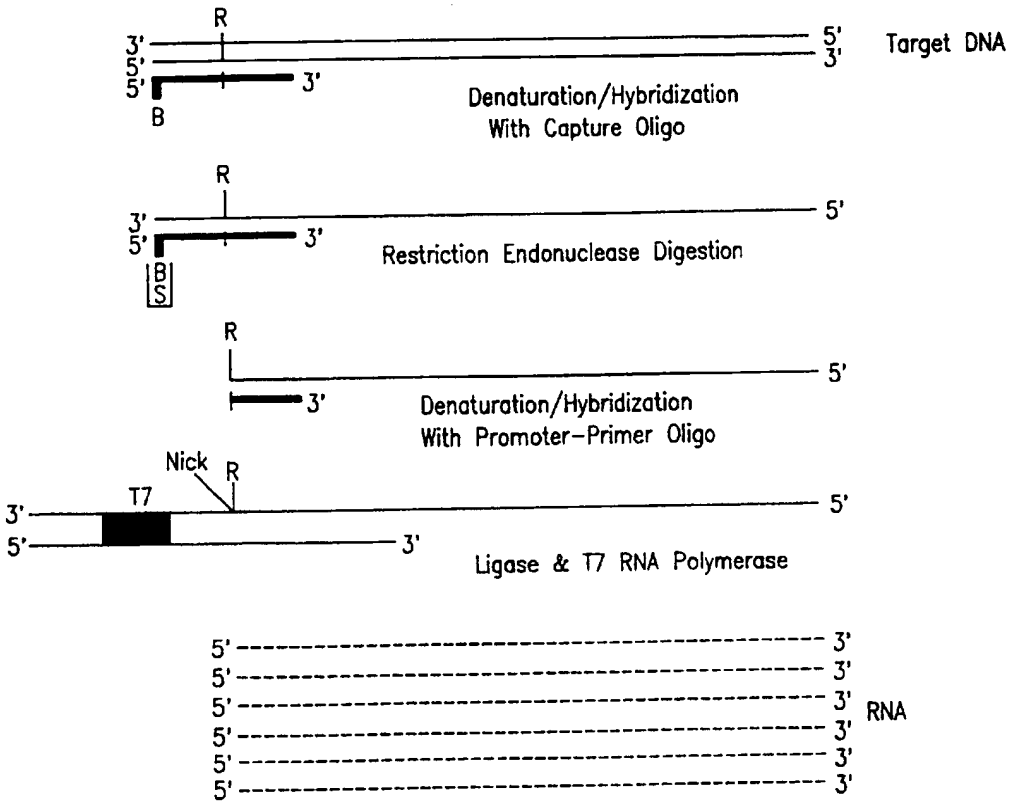
Synthesis

FIG. 4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5'
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With Capture Oligo
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5'
5'
3'
B
S
Restriction Endonuclease Digestion
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5'
3'
Denaturation/Hybridization
With Promoter-Primer Oligo
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3'
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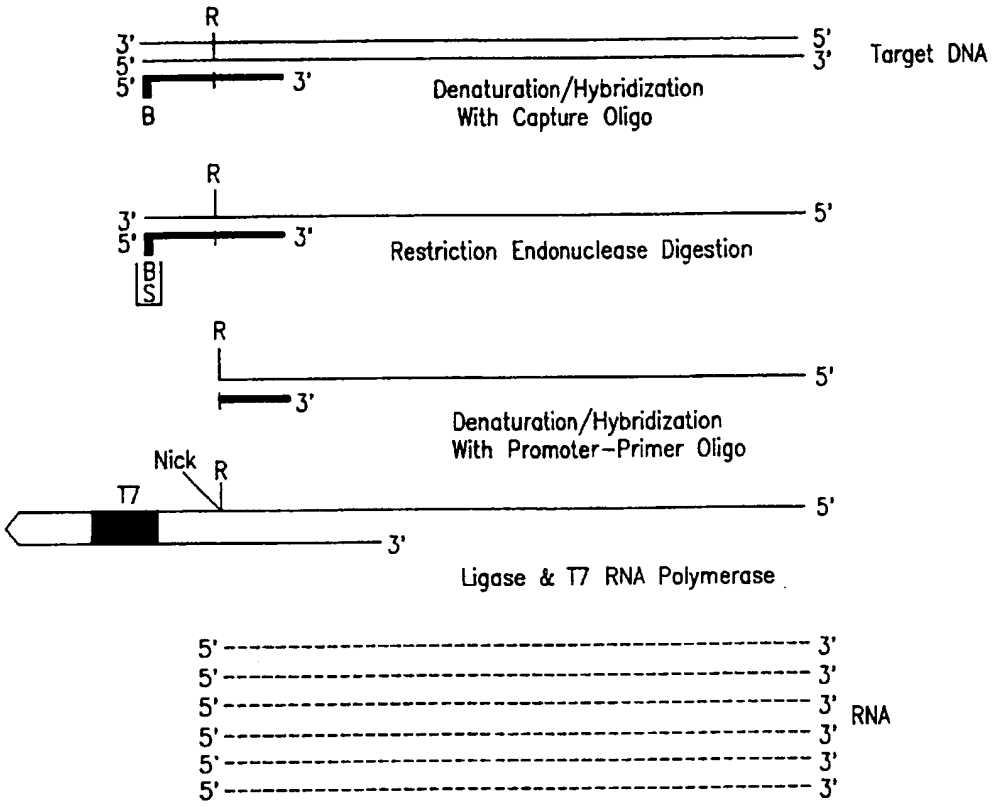
3'
RNA

FIG. 5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5'
3'
B
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Restriction Endonuclease Digestion
R
3'
Denaturation/Hybridization
With Promoter-Primer Oligo
Nick
R
T7
3'
Ligase & T7 RNA Polymerase
RNA

FIG. 6

INTERNATIONAL SEARCH REPORT

Interna ul Application No
PCT/US 96/14806

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO,A,89 06700 (GENENTECH INC) 27 July 1989 see the whole document ---	1-13,26, 27 20-25, 28-32
Y	WO,A,93 10263 (DGI INC) 27 May 1993 see the whole document ---	21-25, 28-32
Y	WO,A,95 08626 (UNIV COLORADO) 30 March 1995 see Fig.4, Example 8 see the whole document ---	20
X	WO,A,91 04340 (CAMBRIDGE BIOTECH CORP) 4 April 1991 see Fig.3 see the whole document --- -/--	1-19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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18 December 1996	17. 01. 97

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INTERNATIONAL SEARCH REPORT

Interna' l Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,93 05184 (LOVE JACK D) 18 March 1993 see the whole document ---	1-32
A	WO,A,93 24658 (GEN TRAK INC) 9 December 1993 see the whole document ---	1-32
A	WO,A,94 03472 (GEN PROBE INC) 17 February 1994 see the whole document -----	1-32

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